

Review

Modifying Bananas: From Transgenics to Organics?

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Abstract: Bananas are one of the top ten world food crops. Unlike most other major food crops, bananas are difficult to genetically improve. The challenge is that nearly all banana cultivars and landraces are triploids, with high levels of male and female infertility. There are a number of international conventional breeding programs and many of these are developing new cultivars. However, it is virtually impossible to backcross bananas, thus excluding the possibility of introgressing new traits into a current cultivar. The alternative strategy is to “modify” the cultivar itself. We have been developing the capacity to modify Cavendish bananas and other cultivars for both disease resistance and enhanced fruit quality. Initially, we were using transgenes; genes that were derived from species outside of the *Musa* or banana genus. However, we have recently incorporated two banana genes (cisgenes) into Cavendish; one to enhance the level of pro-vitamin A and the other to increase the resistance to Panama disease. Modified Cavendish with these cisgenes have been employed in a field trial. Almost certainly, the next advance will be to edit the Cavendish genome, to generate the desired traits. As these banana cultivars are essentially sterile, transgene flow and the outcrossing of modified genes into wild *Musa* species. are highly unlikely and virtually impossible in other triploid cultivars. Therefore, genetic changes in bananas may be compatible with organic farming.

Keywords: bananas; disease resistance; biofortification; organic farming; genetic modification

1. About Bananas

Bananas are a fascinating crop. They are grown almost exclusively in the wet tropics and sub-tropics, but are consumed in nearly every country, as the world’s most popular fruit. However, they are much more than that. Unlike the other nine of the world’s top 10 food crops, bananas are eaten both raw and cooked, depending on the cultivar. About 60% of bananas are eaten raw, as a dessert fruit, and the other 40% are cooked during processes steaming, boiling, roasting, and frying. These cooked bananas include the East African Highland bananas and the African and Pacific plantains, and are extremely important starch sources in Africa and Asia. In some countries, such as Uganda, cooking bananas are the staple food, where consumption levels are in excess of 500 g per person per day. More than 120 million tonnes of banana fruit are produced each year, with the three biggest producers, India, Uganda, and China, consuming almost all of what they produce domestically [1]. About 85% of worldwide banana production is consumed in-country. This domestic production and consumption is made up of many different cultivars; for dessert bananas, Cavendish is very common in many countries, but there are also popular local dessert cultivars, such as Lakatan in the Philippines, Rasthali in India, Gros Michel and Sukali Ndizi in Uganda, and Prata in Brazil. In contrast, the 15% of export bananas, those consumed in developed countries, is almost exclusively one dessert cultivar, Cavendish. The large exporting countries are primarily in Latin America and include Ecuador,

Colombia, Costa Rica, Guatemala, and Honduras [1]. About 15% of the export bananas are grown in the Philippines. It is estimated that an excess of 40% of the world's total banana production is also comprised of just one cultivar, Cavendish [2,3]. However, bananas as a starch source are assuming a greater importance as a food security crop, particularly in Africa. Bananas are perennial and while they require a significant level of rainfall to produce bunches, they can withstand long periods with little water, without dying. Following rainfall, the bananas resume normal growth and produce fruit. This is a tremendous advantage in the increasingly erratic climatic patterns in Africa, where annual crops may fail during prolonged droughts [4].

Bananas have been cultivated from very early on in the development of agriculture, with the earliest record from New Guinea, about 7000 years ago [5–7]. Virtually all cultivated bananas are either diploid or triploid hybrids, between sub-species of *Musa acuminata*, or between *M. acuminata* diploids and *M. balbisiana*. The centre of origin is located from South Asia across to South East Asia, through Papua New Guinea and the northern tip of Australia. The key to their success as an agricultural crop is that these edible diploids and triploids are parthenocarpic, producing seedless fruit. They also have very low levels of male and female fertility. With very few exceptions, the cultivars grown today are probably the result of selections made thousands of years ago [7]. Unfortunately, the key to their success and popularity is also their “Achilles heel”. As the cultivars are parthenocarpic and have a low fertility, they have been vegetatively propagated using suckers since the time of their selection, and thus, they have not been genetically improved, unlike most other major crops. There has clearly been some somatic mutation, but this has only had minor impacts on genetic diversity. This is exemplified within the Cavendish sub-group, where there are numerous clones such as Grand Nain, Williams, Dwarf Cavendish, and Valery, which have been selected for particular traits such as height. Essentially, cultivated bananas have probably been in a state of genetic limbo for thousands of years. Couple this with the dependence of much of the global production on very few cultivars, and an environment has been created where there is potential for catastrophic outcomes. This has already happened. While Cavendish is the extreme example of dependence on a single dominant genotype, this wasn't always the case. In the first half of the last century, Gros Michel was the dominant export cultivar. However, it soon became evident that Gros Michel was highly susceptible to a soil-borne disease called Panama disease, or Fusarium wilt. This disease, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) race 1, spread rapidly across South and Central America [8] in an epidemic that rivals the impact of the late blight epidemic in Ireland; the cause of the great potato famine. There were no chemical controls and the fungus remained active in the soil, probably indefinitely. The demise of Gros Michel led to the wide adoption of the Foc race 1 resistant cultivar, Cavendish. Foc race 1 is now endemic in virtually all banana-producing countries and continues to limit the production of susceptible, non-Gros Michel cultivars, of which there are many.

2. The Challenges and Opportunities for Future Genetic Improvement

The challenges: Diseases are the major constraint to banana production worldwide. While the Panama disease (Fusarium wilt) epidemic in Gros Michel was the first major banana calamity, it is certainly not the last. Currently, there are four major diseases causing very serious concerns and losses: Fusarium wilt tropical race 4 (TR4), black Sigatoka, banana bunchy top disease, and banana Xanthomonas wilt (BXW). There are also larger number of diseases and pests of lesser importance, including yellow Sigatoka, freckle, banana bract mosaic, banana streak, Moko, blood disease, nematodes, and weevils [9].

The most serious threat is Fusarium wilt TR4 [8,10] (Figure 1A–C). TR4 differs from the disease that decimated Gros Michel last century, in that it infects and kills Cavendish, as well as a number of other race 1 resistant cultivars. As such, TR4 has the capacity to kill more than 50% of the bananas grown in the world today. The disease is similar to race 1, in that it is soil-borne, there are no chemical controls, and once the soil becomes infested the fungus can survive for decades [11,12]. It is spread by infected plants, soil on boots, clothing, machinery, and in water, including irrigation and flood water.

Believed to have originated in Indonesia [13], it is now widespread in south-east Asia, including China, Taiwan, and the Philippines [8], where it is threatening both the Cavendish export industry and the local Lakatan production [14]. It is also present in northern Australia [8,15]. Additionally, this disease is on the move. It has recently been recorded in Pakistan near the Indian border [16], Lebanon [17] and Jordan in the Middle East [18], and Mozambique in Africa [19]. Some tolerant, but not resistant, selections from Cavendish have been reported [20].



Figure 1. Banana plants are susceptible to several fungal and viral diseases. (A) External symptoms; and (B,C) characteristic internal vascular discoloration caused by *Fusarium wilt tropical race 4* (TR4); (D) Characteristic symptoms of banana bunchy top disease.

Black Sigatoka, caused by the air-borne fungus *Mycosphaerella fijiensis* (Morelet), severely affects bananas in most producing countries [21]. It can be controlled by the application of fungicides, but often requires spraying more than once a week. This may cause serious long term environmental damage. Spraying is usually limited to commercial production operations, where the cost of control can be a third of the production costs. Smallholder and subsistence farmers rarely have access to these fungicides and must accept the production losses, and with it, the loss of food and income.

Bunchy top (Figure 1D), caused by *Banana bunchy top virus* (BBTV), is widespread in Asia and Africa, but not present in the Americas [22,23]. Spread by aphids and via infected planting material, the disease is aggressively moving through Africa, recently destroying the smallholder-based Cavendish industry in Malawi and becoming established in Nigerian plantains [24–26]. Importantly, the disease can be controlled, but cannot be eradicated in well organised commercial settings through the use of virus-free planting material and the regular plantation inspection and eradication of infected plants. These schemes are rarely implementable in smallholder and subsistence farming environments.

Banana Xanthomonas wilt (BXW), caused by the bacterium *Xanthomonas campestris* pv. *musacearum*, has been responsible for major losses in the past decade in East Africa, after apparently moving from Ethiopia to Uganda, Rwanda, Burundi, Democratic Republic of Congo, Tanzania, and Kenya [25]. The bacterium is transmitted through the soil, insects, and on contaminated implements [27]. Control is effective through the sterilisation of work tools, destruction of infected plants, and fallow of contaminated land. However, maintaining the necessary level of disease management in smallholder and subsistence farming environments is proving to be challenging.

For these four diseases, genetic resistance is the most appropriate control strategy.

Other opportunities: The potential for the genetic improvement of bananas is not limited to disease resistance. Improving the nutritional value of bananas has become a prime target, particularly where cooked bananas are consumed as one of the dietary staples. In most developing countries, the “poorest

of the poor” are becoming increasingly dependent on one or a few high starch staples, including rice, maize, cassava, wheat, sorghum, and bananas. Invariably, these staples are low in essential micronutrients, such as pro-vitamin A and iron. The levels of vitamin A deficiency (VAD) and iron deficiency anaemia (IDA) in most developing countries are unacceptably high. For instance, in Uganda, where bananas are the staple food, vitamin A deficiency is between 15% and 30% in children under 60 months, and about the same for women of a child-bearing age [28,29]. The levels of IDA are worse and are persisting in Uganda and other African countries, despite strategies of food fortification and supplements being successfully implemented in other parts of the world. Biofortification, the process of increasing the levels of essential nutrients in particularly staple foods, is now being developed as an additional strategy to tackle these micronutrient deficiencies, and in a way, this does not require continual external inputs [30–32]. The most advanced of the biofortification by genetic modification (GM) projects is Golden Rice (<http://www.goldenrice.org/>). This program, as well as BioCassavaPlus (<https://www.danforthcenter.org/scientists-research/research-institutes/institute-for-international-crop-improvement/crop-improvement-projects/biocassava-plus>) and Banana21 (<http://www.banana21.org/>), were funded by the Bill & Melinda Gates Foundation as part of the Grand Challenges in Global Health initiative.

There are additional opportunities, such as: increasing fruit shelf life [33]; increasing abiotic stress tolerance, including drought tolerance [34]; and enhancing bananas as a functional food, for instance by producing banana fruit with increased levels of vitamin C [35], which is likely to increase iron bioavailability.

3. Conventional Breeding or Genetic Modification

Despite cultivated bananas having low levels of male and female fertility, it is possible to breed bananas using conventional techniques. There are a number of internationally established breeding programs, usually with an emphasis on different banana types (dessert or cooking) and traits. The FHIA program in Honduras (<http://www.fhia.org.hn/>) has been in operation for many decades and has released a number of hybrids; some of which have important disease resistances, such as black Sigatoka resistance and Fusarium wilt resistance. The EMBRAPA (Brazil, <https://www.embrapa.br/>) and CIRAD (Guadeloupe, <http://www.cirad.fr/en>) programs are also concentrating on generating new hybrids with disease resistances. The IITA (Nigeria and Tanzania, <http://www.iita.org/>) and the NARO (Uganda, <http://www.naro.go.ug/>) programs also target disease resistance and concentrate on developing new cooking banana and plantain cultivars.

The conventional banana breeding programs depend on utilising fertile diploids to hybridise either triploid cultivars or tetraploid intermediates. This has been a successful strategy. However, it has two limitations. The first is that, because of the low male and female fertility, it is virtually impossible to repeatedly backcross the initial hybrid to the original cultivar and therefore, introgressing new traits into the accepted cultivars is also virtually impossible. The second limitation is that not all of the major desirable traits are available in the fertile diploids and thus, these traits cannot be included in conventional breeding programs. For instance, fertile diploids with resistance to black Sigatoka and/or Fusarium wilt race 1 and TR4 are available and have been exploited very effectively [36–40]. However, fertile diploids with resistance to banana bunchy top or banana *Xanthomonas* wilt, have not been identified.

A third and unexpected limitation of conventional banana breeding relates to one of the major progenitors of cultivated bananas, *Musa balbisiana*. This species contributes disease resistance and abiotic stress tolerance. At least one copy of its genome is present in many of the major cultivated bananas, including the plantains, the cooking bananas Saba and Bluggoe, and most of the locally popular acid sweet dessert bananas. The genome of *M. balbisiana*, however, contains integrated sequences of the pararetrovirus *Banana streak virus* (BSV), which upon hybridisation, can recombine to form infectious BSV and symptoms of that infection [41]. This discovery has limited the use of parents containing a *M. balbisiana* genome, thereby severely reducing the available banana breeders’ gene pool.

In contrast, GM can be applied to add a single gene or a few genes to an already highly desirable cultivar. This is extremely important in bananas. As previously noted, Cavendish accounts for more than 40% of the world's banana production. It is so amazingly popular because, for producers, it is high yielding and has excellent transportability and, for the consumer, it has a highly desirable texture and flavour. It excels as a cultivar for providing "cheap fruit" to the world market. No other known selection or conventionally bred cultivar has this combination of characteristics; there is no known replacement for Cavendish. Unfortunately, Cavendish is susceptible to black Sigatoka, banana bunchy top, banana Xanthomonas wilt, and Fusarium wilt TR4 [19–22]. A further example is the East African Highland bananas, which are grown extensively in Uganda, Rwanda, and the Democratic Republic of Congo, as well as Kenya and Tanzania. These cooking bananas probably account for more than 10% of the world's production [42]. More than a staple food, these bananas are an integral part of the culture, particularly in Uganda. The flavour, texture, and "processability" are paramount, and not easily replicated in new cultivars. Unfortunately, these bananas are susceptible to black Sigatoka, banana bunchy top, and banana Xanthomonas wilt. There are many other examples: Lakatan in the Philippines is the dessert banana of choice in a country that produces 15% of the exported Cavendish bananas. Lakatan is susceptible to Fusarium wilt TR4 and banana bunchy top [43].

The accessible gene pool available for GM is significantly broader than that available to conventional banana breeders and, as a result, genes for resistance to BBTV and BXW have already been identified [44–47]. Further, genes from *M. balbisiana* are available for use in genetic modification, as there is no possibility of transferring BSV genes [48].

Similar to conventional breeding with the fertile diploids as a source of desired traits, GM of bananas depends on transformable tissue and genes for the target trait. The transformable tissue of choice for bananas is embryogenic cell suspensions derived from immature male flowers [49]. While the development of these cell suspensions is a lengthy process (Figure 2), it is probable that, with sufficient effort, it should be possible to generate them from most cultivars. In our laboratory, we have generated transformable cell suspensions from Cavendish, Gros Michel, Lady finger, and Goldfinger. Other cultivars from laboratories include the East African Highland banana, Gonja (plantain), and Rasthali. Additionally, efficient transformation protocols are available for bananas, for both *Agrobacterium*-mediated transformation and microprojectile bombardment [49–51]. There are a number of laboratories around the world that have established banana transformation pipelines, including ours at Queensland University of Technology (<https://www.qut.edu.au/>), the IITA in Kenya (<http://newint.iita.org/>), NARO in Uganda (<http://www.naro.go.ug/>), the Bhabha Atomic Research Centre in India (<http://www.barc.gov.in/>), and the Catholic University of Leuven in Belgium (<https://www.kuleuven.be/english>).

Very importantly, genes that can be mobilised into genetically modified bananas are becoming increasingly available, and it is likely that the availability of suitable genes will grow significantly. There are, and will be, two major sources: banana genes and genes from other sources, particularly from other plant species or genes that trigger natural mechanisms in bananas, as well as other plant species. There is, of course, the potential to mobilise bacterial genes for traits such as herbicide tolerance and insect resistance, but this certainly has not been a priority for bananas. The first banana genome sequence was published in 2012 [52]; this sequence is of a double haploid *M. acuminata* ssp. *malaccensis* and has immediately become a major resource. Genes for the resistance to Fusarium wilt TR4 and black Sigatoka exist in *M. acuminata* ssp. *malaccensis*. The genome sequence of *M. balbisiana* have also been published [53], as well as numerous transcriptomic data from various banana tissues [54–56]. In parallel, there are mapping programs to identify loci for various traits, such as resistance to Fusarium wilt TR4. Coupled with the genome sequences, these programs should result in the definitive identification of the gene or genes responsible for specific traits. Further, new "generic" technologies are being developed or refined, and can be applied to the GM of bananas. These include RNA interference technologies, an understanding of the different resistance and other metabolic pathways in plants,

and gene editing technologies such as CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins) [57,58].

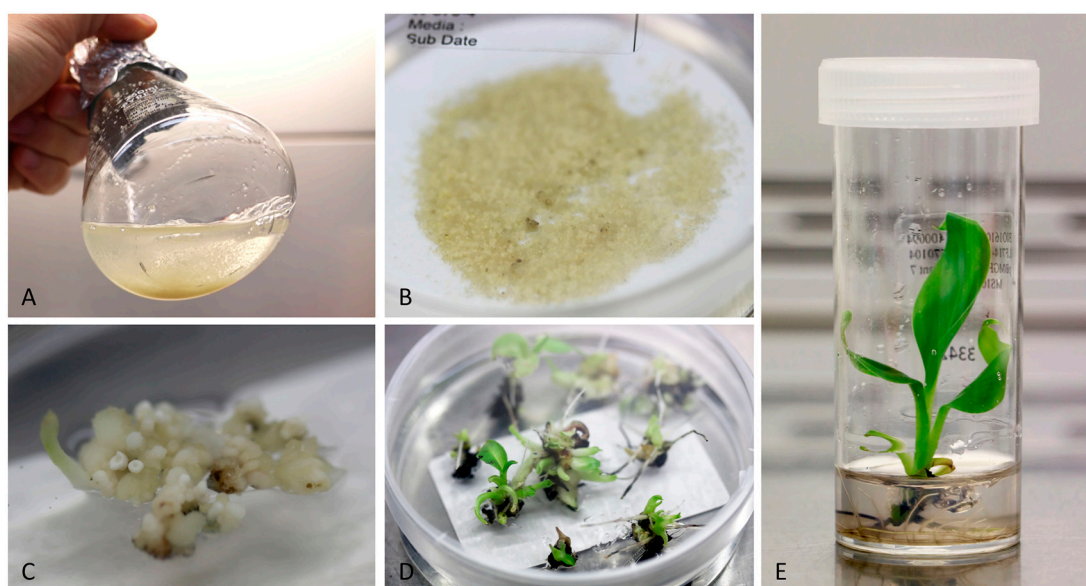


Figure 2. Schematic representation of the genetic transformation system in banana from embryogenic cell suspensions (ECS) through to generation of a genetically modified (transgenic) plant. (A) Banana ECS are mixed with *Agrobacterium* containing the “gene of interest” (GOI), a process which allows the GOI to be transferred (transformed) into banana cells; (B) Cells are transferred to semi-solid selection media to kill non-transformed cells; (C) Cells containing the GOI are induced to form embryos which (D) germinate into transgenic plants; (E) Individual plantlets are transferred to different media to form roots.

4. The Progress with Genetically Modified Bananas

There has been a dramatic increase in the number of published reports of genetically modified bananas over the past five years, with a multitude of different genes targeting different traits. The majority of these studies have been reports from a glasshouse evaluation and therefore, it is difficult to determine whether they only represent experimental projects, or whether they are part of a program to progress to field trial, and ultimately, to general release. The most targeted traits have been pest and disease resistance, abiotic stress tolerance, and fruit ripening, and these have included resistance to BBTV using RNAi (RNA interference) [44,59], resistance to *Fusarium* wilt race 1 using anti-apoptosis genes [60], RNAi [61] or a defensin gene [62], resistance to nematodes using plant-derived or synthetic peptides [63], and the over-expression of a banana-derived WRKY transcription factor to alter abiotic responses [64]. These reports, in addition to a number of others, have used a range of banana genes, RNAi constructs derived from banana pathogens, or genes not derived from bananas.

Of greater interest are the genetically modified bananas that have progressed through to field trials. There were at least two early field trials which both assessed black Sigatoka resistance, but neither of these appear to have progressed further than the initial trial. The first of the more recent field trials commenced in 2009 in Australia, as part of a Bill & Melinda Gates Foundation funded Grand Challenges in Global Health project, to develop cooking bananas for Uganda with enhanced levels of pro-vitamin A (PVA) and iron [31]. As previously stated, VAD and IDA remain major public health problems in East Africa, despite the widespread intervention of supplements and food fortification. The biofortification approach aims to generate East African Highland bananas, the staple food of Uganda, with high levels of PVA. The project is a collaboration between Queensland University of Technology in Australia

and the National Agricultural Research Organisation of Uganda. The initial Australian field trial was in Cavendish (Figure 3A) to identify a strategy that could be transferred to Uganda, to transform into the Ugandan cultivars. From this and successive Australian field trials, two promoters were identified, together with one transgene, that provided enhanced levels of PVA (Figure 3B) nearly three-fold over target, with a stable expression over more than five years. These gene constructs have been transferred to Uganda and have been transformed into the East African Highland banana clone, Nakitembe, as well as a conventionally bred cooking banana, Kabana 6H. The resultant lines are in the field in Uganda, with the aim of selecting elite lines to progress through to the deregulation and release to Ugandan farmers [James Dale, unpublished data]. Most importantly, the selected transgene is a phytoene synthase gene from a F'ei banana called Asupina (Figure 3C). This banana, a native of Papua New Guinea, is naturally very high in PVA but has a small bunch, long maturity time, and is very different in taste and texture to East African Highland bananas. Both gene constructs contain plant-derived promoters and one of those is derived from bananas. It is therefore feasible to develop an intragenic line using this gene construct. East African Highland bananas, like Cavendish, also contain a phytoene synthase gene similar to the Asupina gene. Perhaps a future alternative strategy would be to up-regulate this gene by targeting its regulatory sequences using a gene-editing strategy such as CRISPR-Cas9, which may result in a non-transgenic, high PVA banana.



Figure 3. Bananas can be genetically modified to accumulate elevated levels of pro-vitamin A (PVA) in fruit. (A) Field trial of PVA-biofortified “Cavendish” banana plants in Australia; (B) PVA-biofortified fruit (left) compared to wild-type fruit (right); (C) F'i type bananas, such as “Asupina”, naturally accumulate high levels of PVA and are a source of useful genes for biofortification.

Banana *Xanthomonas* wilt has caused massive losses in East Africa. There are no accessible BXW resistance genes available and GM was clearly the most appropriate strategy to curtail this devastating disease. A team from the International Institute for Tropical Agriculture and the National Agricultural Research Organisation of Uganda have utilised two genes from sweet pepper that are involved in enhancing bacterial resistance, to develop genetically modified bananas and progress these through to the field. The two genes, the hypersensitive response-assisting protein gene (*Hrap*) and the plant

ferredoxin-like protein gene (*Pflp*), were transformed into the dessert banana Sukali Ndizi, under the control of a plant-derived constitutive promoter. The transgenic lines were screened in the glasshouse, before progressing to the field. Lines with either transgene were identified with an apparently complete resistance to BXW [45–47]. The program has progressed further as the two genes, either alone or in combination, have been transformed into Nakitembe, the East African Highland banana clone used in the PVA project. Promising lines are now progressing into field trials in Uganda, again with the intention of selecting elite lines that will be deregulated and released to farmers [65].

There exists the potential to combine both enhanced PVA and BXW resistance in the one genetically modified line for East African Highland bananas in Uganda. This would be very attractive, as it combines two field-proven GM strategies, with one targeting resistance to a major disease constraint and the other targeting a major micronutrient deficiency.

In January, 2012, we planted a field trial in northern Australia to test the resistance to Fusarium wilt TR4 in Cavendish genetically modified with either an anti-apoptosis gene, *Ced9* [60], or a nucleotide binding site-leucine rich repeat (NBS-LRR) type resistance (R) gene isolated from a resistant seedling of *Musa acuminata* ssp. *malaccensis* [66,67], in an area that had been devastated by TR4. The trial ran for three years and the outcomes will be published shortly. Very recently, results from a field trial of Cavendish bananas with delayed fruit ripening were reported [33]. These researchers had down-regulated the expression of a banana MADS box gene homologous to the tomato *ripening inhibitor* (RIN)-MADS ripening gene using RNAi, and demonstrated both ripening delay and extended shelf life. Finally, we have generated a large number of Cavendish lines with a range of RNAi constructs targeting different BBTV genes, that have shown levels of BBTV resistance in the glasshouse in Australia [59]. Many of these lines have been progressed through to a field trial recently planted in Malawi. The RNAi constructs specifically target the South Pacific strain of BBTV, which is the only strain reported from Africa [26].

5. Banana GM and Organic Farming

Many of the principles of organic farming overlap with the primary principles of the major banana biotechnology programs: elimination of pesticides and the sustainable production of traditional and “heirloom” cultivars and landraces, in the case of biotechnology, through the genetic improvement of these cultivars and landraces. Bananas are very different to the broadacre genetically modified commodity crops such as soybeans, maize, cotton, and canola. Only 15% of banana production is exported. This production is based almost exclusively on Cavendish, and is primarily monoculture plantation production with high levels of pesticide usage to control nematodes and fungal diseases. In contrast, the majority of the other 85% of bananas are produced by small holder and subsistence farmers in developing countries and most of these bananas are consumed locally, either on the farm or within a few kilometres. It is likely that a very significant proportion of these bananas are grown without inorganic fertilisers and without the application of pesticides or herbicides. Thus, many bananas grown and consumed in the tropics and sub-tropics are, by circumstance, “organic”. It is not that these bananas are unaffected by pests and diseases; they are. Depending on the geographical location, these include weevils, black and yellow Sigatoka, Fusarium wilt race 1 and TR4, BBTV, and bacterial wilts such as Blood disease, Moko, and BXW. The resources required to control these biotic stresses are invariably unavailable to the small holder farmers and subsequent losses are inevitable and endured. The potential of GM is to provide resistance to these pests and diseases in the cultivars and landraces that are traditionally produced by these farmers. Under current definitions, these genetically modified improved cultivars with disease resistance would no longer be considered as organic, but their production would be significantly more sustainable.

Importantly, while about 25% of the non-export bananas are the dominant Cavendish cultivar, the remaining 75% consist of a wide range of diverse cultivars and landraces, which are highly valued locally. These cultivars and landraces are under as much threat as Cavendish, but receive little international publicity; it is the threat of an end to cheap banana fruit to markets in North

America, Europe, and Japan, that receives the most attention. Although developing a disease-resistant Cavendish or Cavendish replacement is extremely important for both the export trade and local production, just as important is conserving the wonderful diversity seen in local markets throughout the wet tropics and subtropics. As a consequence of the very low fertility of most triploid cultivated bananas, it is essentially impossible to introgress new traits into these cultivars via multiple backcrosses. At present, the genetic improvement, and thus the long term sustainable production of these cultivars, can only be achieved through genetic modification.

The conventional breeding programs are targeting the development of new cultivars with resistance to two of the major diseases, black Sigatoka and Fusarium wilt TR4, as these traits are available to banana breeders within the banana gene pool. The GM programs are targeting the development of resistance to Fusarium wilt TR4, BBTV, and BXW in current cultivars and landraces, with either banana genes, other plant genes, or by triggering natural banana virus resistance mechanisms. No suitable GM approach for black Sigatoka resistance has been identified, but it will come. Clearly, there is an opportunity to incorporate both conventional and GM approaches to optimise new cultivars, but this also is yet to be achieved. Interestingly, the current control regimes for all four of the major diseases of bananas, comprised of black Sigatoka, BXW, banana bunchy top, and Fusarium wilt TR4, involve the use of chemicals. The large scale use of fungicides to control black Sigatoka is well documented [68]. One major element of the current control of Fusarium wilt TR4 is the use of disinfectants for boots and vehicles. Elements for the control of banana bunchy top include herbicides to kill infected plants and insecticides to kill the aphid vectors, while disinfectants are used to sterilise tools for BXW control and herbicides are used to kill infected plants. Genetic resistance to these diseases would dramatically reduce the use of harsh chemicals in control regimes.

The genome revolution is well under way and bananas are not being left behind.

Genes for pest and disease resistances will become available at an ever increasing pace, and the majority of these will come from bananas themselves, drawing on the amazing genetic diversity of both wild and cultivated bananas. Banana genes have already successfully been mobilised using GM for the development of bananas with elevated pro-vitamin A and delayed fruit ripening.

In parallel, new genetic technologies are being developed and are already being adapted to bananas. CRISPR-Cas9 is the first of probably many genome-editing technologies, and groups such as ours are already developing this approach for the next generation of genetically improved bananas. The line between conventionally bred and genetically modified is becoming increasingly blurred, and this will continue. It is likely that smaller labs in the wet tropics and subtropics will develop the capacity to genetically but non-conventionally improve bananas, and it is likely that they will ultimately concentrate on improving their local favourites.

The future of genetically improved bananas looks very positive, if it is allowed to develop unimpeded: this includes the conservation of traditional and heirloom cultivars and landraces, the elimination of pesticides, and a reduction of the impact of micronutrient deficiencies in developing countries, without the risk of transgene escape from modified bananas to other cultivars or to wild-type plants. Moreover, it is likely that these bananas will be developed with the strong input and influence of local organisations. The genetic resources will be broadly held. Bananas are an excellent example where the likely outcomes of the application of GM match with many of the desired outcomes of organic production.

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References

1. Food and Agriculture Organization of the United Nations (FAOSTAT). Available online: <http://www.fao.org/faostat/en/#data/QC> (accessed on 15 August 2016).

2. Ploetz, R.C.; Kepler, A.K.; Daniells, J.; Nelson, S.C. Banana and Plantain: An Overview with Emphasis on Pacific Island Cultivars. In *Species Profiles for Pacific Island Agroforestry*; Elevitch, C.R., Ed.; Permanent Agriculture Resources (PAR): Holualoa, HI, USA, 2007; Available online: <http://agroforestry.org/images/pdfs/Banana-plantain-overview.pdf> (accessed on 9 February 2017).
3. Arias, P.; Dankers, C.; Liu, P.; Pilkauskas, P. Chapter 1: Overview of world banana production and trade. In *The World Banana Economy 1985–2002*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2003.
4. Alliance for a Green Revolution in Africa (AGRA). *Africa Agriculture Status Report 2014: Climate Change and Smallholder Agriculture in Sub-Saharan Africa*; AGRA: Nairobi, Kenya, 2014; Available online: <https://ccaafs.cgiar.org/publications/africa-agriculture-status-report-2014-climate-change-and-smallholder-agriculture-sub#> (accessed on 9 August 2016).
5. Denham, T.; Haberle, S.; Lentfer, C.J.; Fullagar, R.; Field, J.; Therin, M.; Porch, N.; Winsborough, B. Origins of Agriculture at Kuk Swamp in the Highlands of New Guinea. *Science* **2003**, *301*, 189–193. [[CrossRef](#)] [[PubMed](#)]
6. Denham, T.; Haberle, S.; Lentfer, C.J. New evidence and revised interpretations of early agriculture in Highland New Guinea. *Antiquity* **2004**, *78*, 839–857. [[CrossRef](#)]
7. Perrier, X.; De Langhe, E.; Donohue, M.; Lentfer, C.; Vrydaghs, L.; Bakry, F.; Carreel, F.; Hippolyte, I.; Horry, J.P.; Jenny, C.; et al. Multidisciplinary perspectives on banana (*Musa* spp.) domestication. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 11311–11318. [[CrossRef](#)] [[PubMed](#)]
8. Ploetz, R.C. Fusarium wilt of banana. *Phytopathology* **2015**, *105*, 1512–1521. [[CrossRef](#)] [[PubMed](#)]
9. Ploetz, R.C.; Evans, E.A. The Future of Global Banana Production. In *Horticultural Reviews*; Janick, J., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2015; Volume 43, p. 311.
10. Ploetz, R.C. Management of Fusarium wilt of banana: A review with special reference to tropical race 4. *Crop Prot.* **2015**, *73*, 7–15. [[CrossRef](#)]
11. Simmonds, N.W. *Bananas*, 2nd ed.; Longmans: London, UK, 1966.
12. Stover, R.H. *Fusarial Wilt (Panama Disease) of Bananas and Other Musa Species*; CMI: Kew, UK, 1962.
13. Buddenhagen, I. Understanding strain diversity in *Fusarium oxysporum* f. sp. *cubense* and history of introduction of ‘Tropical Race 4’ to better manage banana production. *Acta Hort.* **2009**, *828*, 193–204.
14. Molina, A.; Fabregar, E.; Sinohin, V.G.; Herradura, L.; Fourie, G.; Viljoen, A. Confirmation of Tropical Race 4 of *Fusarium oxysporum* f. sp. *cubense* Infecting Cavendish Bananas in the Philippines. In Proceedings of the Centennial Meeting of the American Phytopathological Society, Minneapolis, MN, USA, 26–28 July 2008; Available online: <https://cgspace.cgiar.org/handle/10568/674> (accessed on 2 August 2016).
15. Queensland Government Department of Agriculture and Fisheries. A–Z List of Emergency Plant Pests and Diseases. Panama Disease. 2015. Available online: <https://www.daf.qld.gov.au/plants/health-pests-diseases/a-z-significant/panama-disease2> (accessed on 1 September 2016).
16. Syed, R.N.; Lodhi, A.M.; Jiskani, M.M.; Rajput, K.I.; Khaskheli, M.A.; Khanzada, M.A.; Rajput, N.A.; Maitlo, S.A.; Rajput, A.Q. First report of Panama wilt disease of banana caused by *Fusarium oxysporum* f. sp. *cubense* in Pakistan. *J. Plant Pathol.* **2015**, *97*, 213. [[CrossRef](#)]
17. Ordonez, N.; Seidl, M.F.; Waalwijk, C.; Drenth, A.; Kilian, A.; Thomma, B.P.; Ploetz, R.C.; Kema, G.H.J. Worse comes to worst: Bananas and Panama disease—When plant and pathogen clones meet. *PLoS Pathog.* **2015**, *11*, e1005197. [[CrossRef](#)] [[PubMed](#)]
18. García-Bastidas, F.; Ordóñez, N.; Konkol, J.; Al-Qasim, M.; Naser, Z.; Abdelwali, M.; Salem, N.; Waalwijk, C.; Ploetz, R.C.; Kema, G.H.J. First report of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 associated with Panama disease of banana outside Southeast Asia. *Plant Dis.* **2013**, *98*, 694. [[CrossRef](#)]
19. Butler, D. Fungus threatens top banana. *Nature* **2013**, *504*, 195–196. [[CrossRef](#)] [[PubMed](#)]
20. Hwang, S.C.; Ko, W.H. Cavendish banana cultivars resistant to Fusarium wilt acquired through somaclonal variation in Taiwan. *Plant Dis.* **2004**, *88*, 580–587. [[CrossRef](#)]
21. Arango Isaza, R.E.; Diaz-Trujillo, C.; Dhillon, B.; Aerts, A.; Carlier, J.; Crane, C.F.; de Jong, T.V.; De Vries, I.; Dietrich, R.; Farmer, A.D.; et al. Combating a Global Threat to a Clonal Crop: Banana Black Sigatoka Pathogen *Pseudocercospora fijiensis* (Synonym *Mycosphaerella fijiensis*) Genomes Reveal Clues for Disease Control. *PLoS Genet.* **2016**, *12*, e1005876. [[CrossRef](#)] [[PubMed](#)]
22. Dale, J.L. Banana bunchy top—An economically important tropical plant virus disease. *Adv. Virus Res.* **1987**, *33*, 301–325. [[PubMed](#)]

23. Harding, R.M.; Burns, T.M.; Dale, J.L. Virus-Like Particles Associated with Banana Bunchy Top Disease Contain Small Single-Stranded DNA. *J. Gen. Virol.* **1991**, *72*, 225–230. [[CrossRef](#)] [[PubMed](#)]
24. Adegbola, R.O.; Ayodeji, O.; Awosusi, O.O.; Atiti, G.I.; Kumar, P. First report of *Banana bunchy top virus* in Banana and Plantain (*Musa* spp.) in Nigeria. *Plant Dis.* **2013**, *97*, 290. [[CrossRef](#)]
25. Blomme, G.; Ploetz, R.; Jones, D.; De Langhe, E.; Price, N.; Gold, C.; Geering, A.; Viljoen, A.; Karamura, D.; Pillay, M.; et al. A historical overview of the appearance and spread of *Musa* pests and pathogens on the African continent: Highlighting the importance of clean *Musa* planting materials and quarantine measures. *Ann. Appl. Biol.* **2013**, *162*, 4–26. [[CrossRef](#)]
26. Kumar, P.L.; Hanna, R.; Alabi, O.J.; Soko, M.M.; Oben, T.T.; Vangu, G.H.P.; Naidu, R.A. Banana bunchy top virus in sub-Saharan Africa: Investigations on virus distribution and diversity. *Virus Res.* **2011**, *159*, 171–182. [[CrossRef](#)] [[PubMed](#)]
27. Eden-Green, S. How can the advance of banana *Xanthomonas* wilt be halted? *InfoMusa* **2004**, *13*, 38–41.
28. Uganda Bureau of Statistics (UBOS). *Uganda Demographic and Health Survey 2006*; UBOS: Kampala, Uganda; Macro International Inc.: Calverton, MD, USA, 2006; Available online: <http://www.dhsprogram.com/pubs/pdf/FR194/FR194.pdf> (accessed on 28 July 2016).
29. Uganda Bureau of Statistics (UBOS). *Uganda Demographic and Health Survey 2011*; UBOS: Kampala, Uganda; MEASURE DHS ICF International: Calverton, MD, USA, 2011; Available online: <https://dhsprogram.com/pubs/pdf/FR264/FR264.pdf> (accessed on 28 July 2016).
30. HarvestPlus. *Disseminating Orange-Fleshed Sweet Potato: Uganda Country Report*; HarvestPlus: Washington, DC, USA, 2012.
31. Dale, J.; Tushemereirwe, W. Development of bananas with optimised bioavailable micronutrients. In *Grand Challenges in Global Health: 2005–2015. A 10-Year Retrospective of 14 of the 44 Original Projects*; 2015; pp. 106–113. Available online: <http://gcgh.grandchallenges.org/sites/default/files/gf%2010%20years%20p7%20111615%20intro.pdf> (accessed on 23 February 2017).
32. Sayre, R.; Beeching, J.R.; Cahoon, E.B.; Egesi, C.; Fauquet, C.; Fellman, J.; Fregene, M.; Gruissem, W.; Mallowa, S.; Manary, M.; et al. The BioCassava Plus program: Biofortification of cassava for Sub-Saharan Africa. *Annu. Rev. Plant Biol.* **2011**, *62*, 251–272. [[CrossRef](#)] [[PubMed](#)]
33. Elitzur, T.; Yakir, E.; Quansah, L.; Zhangjun, F.; Vrebalov, J.; Khayat, E.; Giovannoni, J.J.; Friedman, H. Banana MaMADS transcription factors are necessary for fruit ripening and molecular tools to promote shelf-life and food security. *Plant Physiol.* **2016**, *171*, 380–391. [[CrossRef](#)] [[PubMed](#)]
34. Ahmad, P.; Ashraf, M.; Younis, M.; Hu, X.; Kumar, A.; Akram, N.A.; Al-Qurainy, F. Role of transgenic plants in agriculture and biopharming. *Biotechnol. Adv.* **2012**, *30*, 524–540. [[CrossRef](#)] [[PubMed](#)]
35. Lisko, K.A.; Torres, R.; Harris, R.S.; Belisle, M.; Vaughan, M.M.; Jullian, B.; Chevone, B.I.; Mendes, P.; Nessler, C.L.; Lorence, A. Elevating vitamin C content via overexpression of myo-inositol oxygenase and L-gulonono-1,4-lactone oxidase in *Arabidopsis* leads to enhanced biomass and tolerance to abiotic stresses. *In Vitro Cell Dev. Biol. Plant.* **2013**, *49*, 643–655. [[CrossRef](#)] [[PubMed](#)]
36. Damodaran, T.; Kumar, N.; Kavino, M. Breeding and evaluation of *Musa* hybrids resistant to *Fusarium oxysporum* f. sp. *cubense* race 1. *Fruits* **2009**, *64*, 3–12. [[CrossRef](#)]
37. Li, W.M.; Dita, M.; Wu, W.; Hu, G.B.; Xie, J.H.; Ge, X.J. Resistance sources to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in banana wild relatives. *Plant Pathol.* **2015**, *64*, 1061–1067.
38. Nowakunda, K.; Barekye, A.; Ssali, R.T.; Namaganda, J.; Tushemereirwe, W.K.; Nabulya, G.; Erima, R.; Akankwasa, K.; Hilman, E.; Batte, M.; et al. 'Kiwangaazi' (syn 'KABANA 6H') Black Sigatoka nematode and banana weevil tolerant 'Matooke' hybrid banana released in Uganda. *Hortic. Sci.* **2015**, *50*, 621–623.
39. Ortiz, R.; Vuylsteke, D. Inheritance of black sigatoka disease resistance in plantain-banana (*Musa* spp.) hybrids. *Theor. Appl. Genet.* **1994**, *89*, 146–152. [[CrossRef](#)] [[PubMed](#)]
40. Ssali, R.T.; Kiggundu, A.; Lorenzen, J.; Karamura, E.; Tushemereirwe, W.; Viljoen, A. Inheritance of resistance to *Fusarium oxysporum* f. sp. *cubense* race 1 in bananas. *Euphytica* **2013**, *194*, 425–430. [[CrossRef](#)]
41. Iskra-Caruana, M.L.; Duroy, P.O.; Chabannes, M.; Muller, E. The common evolutionary history of badnaviruses and banana. *Infect. Genet. Evol.* **2014**, *21*, 83–89. [[CrossRef](#)] [[PubMed](#)]
42. Arias, P.; Dankers, C.; Liu, P.; Pilkauskas, P. *The World Banana Economy (1985–2002)*; FAO: Rome, Italy, 2003; Available online: <http://www.fao.org/docrep/007/y5102e/y5102e04.htm> (accessed on 17 August 2016).

43. Banana Market Review and Banana Statistics (2012 and 2013). Food and Agricultural Organization of the United Nations, 2014. Available online: <http://www.fao.org/docrep/019/i3627e/i3627e.pdf> (accessed on 15 August 2016).
44. Shekhawat, U.K.S.; Ganapathi, T.R.; Hadapad, A.B. Transgenic banana plants expressing small interfering RNAs targeted against viral replication initiation gene display high-level resistance to banana bunchy top virus infection. *J. Gen. Virol.* **2012**, *93*, 1804–1813. [[CrossRef](#)] [[PubMed](#)]
45. Namukwaya, B.; Tripathi, L.; Tripathi, J.N.; Arinaitwe, G.; Mukasa, S.B.; Tushemereirwe, W.K. Transgenic banana expressing *Pflp* gene confers enhanced resistance to *Xanthomonas* wilt disease. *Transgen. Res.* **2012**, *21*, 855–865. [[CrossRef](#)] [[PubMed](#)]
46. Tripathi, L.; Mwaka, K.; Tripathi, J.N.; Tushemereirwe, W.K. Expression of sweet pepper *Hrap* gene in banana enhances resistance to *Xanthomonas campestris* pv. *musacearum*. *Mol. Plant Pathol.* **2010**, *11*, 721–731. [[PubMed](#)]
47. Tripathi, L.; Tripathi, J.N.; Kiggundu, A.; Korie, S.; Shotkoski, F.; Tushemereirwe, W.K. Field trial of *Xanthomonas* wilt disease-resistant bananas in East Africa. *Nat. Biotechnol.* **2014**, *32*, 868–870. [[CrossRef](#)] [[PubMed](#)]
48. Umber, M.; Pichaut, J.P.; Farinas, B.; Laboureau, N.; Janzac, B.; Plaisir-Pineau, K.; Pressat, G.; Baurens, F.C.; Chabannes, M.; Duroy, P.O.; et al. Marker-assisted breeding of *Musa balbisiana* genitors devoid of infectious endogenous Banana streak virus sequences. *Mol. Breed.* **2016**, *36*, 1–11. [[CrossRef](#)]
49. Khanna, H.; Becker, D.; Kleidon, J.; Dale, J.L. Centrifugation assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). *Mol. Breed.* **2004**, *14*, 239–252. [[CrossRef](#)]
50. Becker, K.D.; Dugdale, B.; Smith, K.M.; Harding, R.M.; Dale, J.L. Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv ‘Grand Nain’ via microprojectile bombardment. *Plant Cell Rep.* **2000**, *19*, 229–234. [[CrossRef](#)]
51. Ortiz, R.; Swennen, R. From crossbreeding to biotechnology-facilitated improvement of banana and plantain. *Biotechnol. Adv.* **2014**, *32*, 158–169. [[CrossRef](#)] [[PubMed](#)]
52. D’Hont, A.; Denoëud, F.; Aury, J.-M.; Baurens, F.-C.; Carreel, F.; Garsmeur, O.; Noel, B.; Bocs, S.; Droc, G.; Rouard, M. The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* **2012**, *488*, 213–217. [[CrossRef](#)] [[PubMed](#)]
53. Davey, M.W.; Gudimella, R.; Harikrishna, J.A.; Sin, L.W.; Khalid, N.; Keulemans, J. A draft *Musa balbisiana* genome sequence for molecular genetics in polyploid, inter- and intra-specific *Musa* hybrids. *BMC Genom.* **2013**, *14*, 1–20. [[CrossRef](#)] [[PubMed](#)]
54. Asif, M.H.; Lakhwani, D.; Pathak, S.; Gupta, P.; Bag, S.K.; Nath, P.; Trivedi, P.K. Transcriptome analysis of ripe and unripe fruit tissue of banana identifies major metabolic networks involved in fruit ripening process. *BMC Plant Biol.* **2014**, *14*, 1–15. [[CrossRef](#)] [[PubMed](#)]
55. Li, C.; Shao, J.; Wang, Y.; Li, W.; Guo, D.; Yan, B.; Xia, Y.; Peng, M. Analysis of banana transcriptome and global gene expression profiles in banana roots in response to infection by race 1 and tropical race 4 of *Fusarium oxysporum* f. sp. *ubense*. *BMC Genom.* **2013**, *14*, 1–16. [[CrossRef](#)] [[PubMed](#)]
56. Wang, Z.; Zhang, J.; Jia, C.; Liu, J.; Li, Y.; Yin, X.; Xu, B.; Jin, Z. De novo characterization of the banana root transcriptome and analysis of gene expression under *Fusarium oxysporum* f. sp. *ubense* tropical race 4 infection. *BMC Genom.* **2012**, *13*, 650. [[CrossRef](#)]
57. Cardi, T.; Stewart, N., Jr. Progress of targeted genome modification approaches in higher plants. *Plant Cell Rep.* **2016**, *35*, 1401–1416. [[CrossRef](#)] [[PubMed](#)]
58. Paul, J.W., III; Qi, Y. CRISPR/Cas9 for plant genome editing: Accomplishments, problems and prospects. *Plant Cell Rep.* **2016**, *35*, 1417–1427. [[CrossRef](#)] [[PubMed](#)]
59. Mwari, B. Development of Banana Bunchy Top Virus Resistance in Bananas: RNAi Approach. Ph.D. Thesis, Queensland University of Technology, Brisbane, Australia, 2016.
60. Paul, J.-Y.; Becker, D.K.; Dickman, M.B.; Harding, R.M.; Khanna, H.K.; Dale, J.L. Apoptosis-related genes confer resistance to *Fusarium* wilt in transgenic ‘Lady Finger’ bananas. *Plant Biotechnol. J.* **2011**, *9*, 1141–1148. [[CrossRef](#)] [[PubMed](#)]
61. Ghag, S.B.; Shekhawat, U.K.S.; Ganapathi, T.R. Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against *Fusarium* wilt in banana. *Plant Biotechnol. J.* **2014**, *12*, 541–553. [[CrossRef](#)] [[PubMed](#)]

62. Ghag, S.B.; Shekhawat, U.K.S.; Ganapathi, T.R. Transgenic banana plants expressing a *Stellaria media* defensin gene (Sm-AMP-D1) demonstrate improved resistance to *Fusarium oxysporum*. *Plant Cell Tissue Org.* **2014**, *119*, 247–255. [[CrossRef](#)]
63. Tripathi, L.; Babiryé, A.; Roderick, H.; Tripathi, J.N.; Changa, C.; Urwin, P.E.; Tushemereirwe, W.K.; Coyne, D.; Atkinson, H.J. Field resistance of transgenic plantain to nematodes has potential for future African food security. *Nat. Sci. Rep.* **2015**, *5*, 8127. [[CrossRef](#)] [[PubMed](#)]
64. Shekhawat, U.K.S.; Ganapathi, T.R. MusaWRKY71 overexpression in banana plants leads to altered abiotic and biotic stress responses. *PLoS ONE* **2013**, *8*, e75506. [[CrossRef](#)] [[PubMed](#)]
65. Tripathi, L.; (IITA, Nairobi, Kenya). Personal communication, 2016.
66. Peraza-Echeverria, S. Molecular Cloning and Characterisation of Potential Fusarium Resistance Genes in Banana (*Musa acuminata* ssp. *malaccensis*). Ph.D. Thesis, Queensland University of Technology, Brisbane, Australia, 2007.
67. Peraza-Echeverria, S.; Dale, J.L.; Harding, R.M.; Collet, C. Molecular cloning and in silico analysis of potential *Fusarium* resistance genes in banana. *Mol. Breed.* **2009**, *23*, 431–443. [[CrossRef](#)]
68. De Lapeyre De Bellaire, L.; Fouré, E.; Abadie, C.; Carlier, J. Black Leaf Streak Disease is challenging the banana industry. *Fruits* **2010**, *65*, 327–342. [[CrossRef](#)]



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