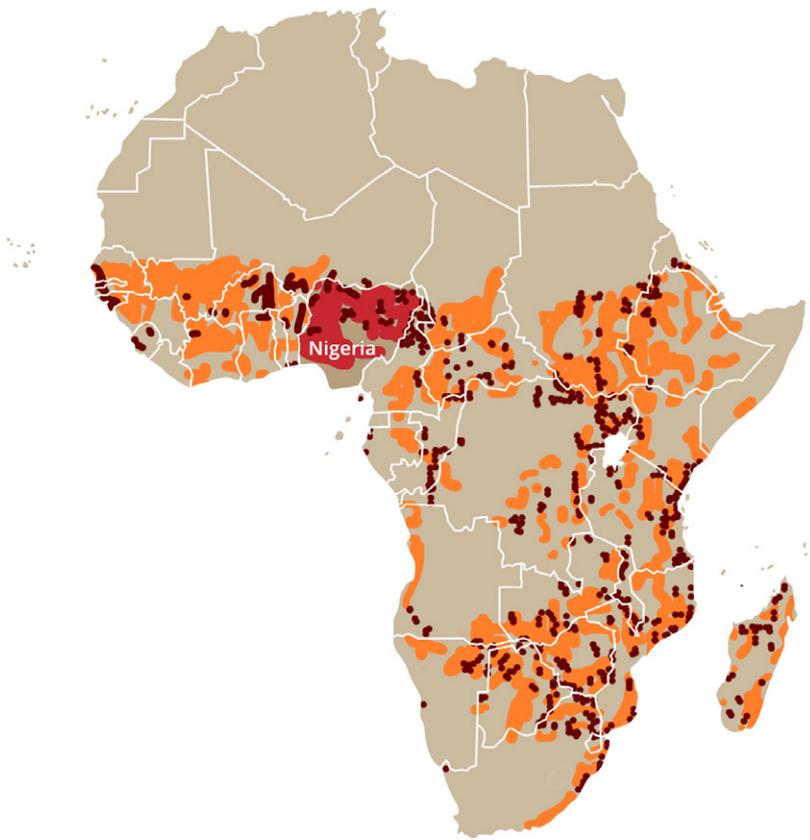TEST
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Cultivation of Bt cowpea may impact health and the environment

Christoph Then, Juliana Miyazaki and Andreas Bauer-Panskus
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Summary

Testbiotech analysed the application for marketing and cultivation of insect-resistant cowpea - Event AAT709A (Ref.No. AAT/DPS-18VUIR-NG) - filed by the African Agricultural Technology Foundation and the Institute of Agricultural Research, Ahmadu Bello University Zaria (AATF, 2018) to the government of the Federal Republic of Nigeria in 2018 and the respective recommendation of the National Biosafety Committee (NBC) of Nigeria (NBMA, 2018) and the decision for approval (NBMA 2019). The transgenic cowpea (*Vigna unguiculata*) produces bacterial toxins originating form Bacillus thuringiensis (Bt toxin) classified as Cry1Ab. The toxins are meant to protect the cowpea against infestations with larvae of *Maruca vitrata* (pod borer).

The aim of the Testbiotech analysis was to come up with a reasoned opinion on whether the application and the recommendation can be regarded as adequate and sufficient to assess the risks of the genetically engineered cowpea, and justify approval for release, cultivation and consumption.

Testbiotech identified and described potential hazards as well as resulting risks which should have been taken into account in the GE cowpea risk assessment. The identified risks were compared to the application and the assessment of the authorities in Nigeria. The issues as presented can also be relevant for other countries in which these GE cowpeas are approved or applied for release or marketing (such as Ghana).

The analysis focused on the following topics:

› stability in the expression of the inserted genes;

› gene flow to other cultivated varieties or wild relatives;

› impact on non-target organisms;

› impact on microbial communities;

› health risks at the stage of consumption.

Specific causes for concern were identified from the available data:

› The expression of the Bt gene appears to lack sufficient stability; it is likely to be influenced by genetic backgrounds (of the specific varieties) and environmental conditions. One probable reason is the specific promoter used to enhance gene expression in the plants.

› Gene flow to regional varieties and wild relatives can be expected from the cultivation of the plants. If gene flow occurs, the GE plants can persist, propagate and generate viable offspring that cannot be controlled. This may trigger adverse effects caused by the spontaneous offspring that were not observed in the original GE cowpea. Long-term risk assessment of these next generation effects cannot be conducted in a sufficiently reliable way.

› The consequences of gene flow for seed collections, seed saving and traditional farming can be severe, putting at risk the common heritage of African farmers.

› The toxicity of Bt proteins (Cry1Ab) is increased by synergistic effects arising from the combination with the protease inhibitors (PI) produced by cowpea. The enhanced toxicity may also impact the selectivity of the toxin and susceptibility of non-target organisms.

› The synergistic effects arising from the combination of Bt toxins with the plant protease inhibitors (PI) may also enhance immune responses, including responses to allergens potentially produced in the cowpea.

› Bt toxins expressed in the plants may also affect their associated microbiome, including endophytes, mycorrhizae and other soil organisms. Negative effects on the microbial properties and the enzymatic activities in the rhizosphere can not be excluded after long-term cultivation.

The concerns raised have a reasonable degree of probability and plausibility. Nevertheless, risk assessment failed to sufficiently address these issues.

Moreover, it appears that the applicant does not acknowledge existing evidence such as:

> the expression of the Bt gene appears to lack sufficient stability;

> the high likelihood of gene flow to wild relatives and regional varieties;

> the immune responses that can be enhanced by Bt toxins;

> synergistic effects between protease inhibitors (PI, as naturally produced in the cowpea) and Bt toxins can substantially increase the toxicity of the cowpeas and the products derived off;

> negative effects on the microbial properties and the enzymatic activities in the rhizosphere may occur after long-term cultivation.

In conclusion, the Bt cowpea risk assessment is inadequate, unreliable and to some extent misleading. The occurrence of adverse effects from the cultivation of the GE cowpea is not unlikely, including impacts on (i) the environment and biodiversity; (ii) the cowpea gene pool; (iii) the livelihoods of farmers as well as (iv) human and animal health. Since these causes for concern cannot be resolved or dispelled by the data presented for risk assessment, the cultivation of the transgenic cowpea in Nigeria should be discontinued.

Introduction

Testbiotech is an independent institute for impact assessment of biotechnology and genetically engineered (GE) organisms. Testbiotech is entirely free of any interest in the development and application of GE organisms, and has extensive experience regarding the risk assessment of GE plants conducted in the European Union. More information about our activities in this field can be found in our database (www.testbiotech.org/en/database) and our publications (www.testbiotech.org/en/publikationen).

Objective of the analysis

Testbiotech analysed the application for marketing and cultivation of insect-resistant cowpea - Event AAT709A (Ref.No. AAT/DPS-18VUIR-NG) - filed by the African Agricultural Technology Foundation and the Institute of Agricultural Research, Ahmadu Bello University Zaria (AATF, 2018) to the government of the Federal Republic of Nigeria in 2018 as well as the respective assessment of the National Biosafety Committee (NBC) of Nigeria (NBMA, 2018). The transgenic cowpea (*Vigna unguiculata*) produces bacterial toxins originating form *Bacillus thuringiensis* (Bt toxin) classified as Cry1Ab. The toxins are meant to protect the cowpea against damage caused by larvae of *Maruca vitrata* (pod borer). The Bt cowpea project, from the beginning, was developed in close interaction between AATF and companies such as Monsanto (AATF, 2008).

The aim of the analysis was to come up with a reasoned opinion on whether the application and the recommendation can be regarded as adequate and sufficient to assess the risks of the GE cowpea, and justify approval for release, cultivation and consumption.

Methodology

Testbiotech analyzed the publicly available data (AATF, 2018), by following the standards established in the EU for the risk assessment of genetically modified organism (GMO) market approval (EFSA, 2010; EU Commission 2013). We started evaluating the biological characteristics of the recipient plants (taking into account gene flow), the description of the intended genetic modifications, the description of the plant components and known environmental interactions of the plants with the environment, including the food webs and non-target organisms. Given the importance of cowpea in the diets of millions of people, the analysis also included considerations concerning health aspects at the stage of consumption. Testbiotech identified and described potential hazards and resulting risks that should have been taken into account in the GE cowpea risk assessment. The identified risks were compared to the application and the assessment made by the Nigerian authorities (NBMA, 2018).

A. Identification and description of hazards and risks

Testbiotech focused on the following topics:

> stability in the expression of the inserted genes;
> gene flow to other cultivated varieties or wild relatives;
> impact on non-target organisms;
> impact on microbial communities;
> health risks at the stage of consumption.

Expression of the genes inserted

The Bt cowpea was engineered to express two gene products. The transgenic cowpea produces bacterial toxins originating form *Bacillus thuringiensis* (Bt toxin) classified as Cry1Ab. The toxins are meant to protect the cowpea against damage caused by larvae of *Maruca vitrata* (pod borer). In addition, the plants express an enzyme which confers resistance to antibiotics (NptII).

Agrobacterium tumefaciens was used for the transformation of the plants; therefore, the site of the insertion, the number of inserted copies and the final structure of the DNA inserted cannot be predicted (Gelvin, 2017). Consequently, each 'event' resulting from the process of transgenesis has to be assessed case by case, taking into account the gene construct, the number of inserted copies, the place of insertion, the expression of the transgene, the stability of its function and its inheritance in offspring generations.

Additionally, the genetic engineering techniques can cause specific unintended effects (see Forsbach et al., 2003; Kim et al., 2003; Latham et al., 2006; Makarevitch et al., 2003; Rang et al., 2005; Windels et al., 2003) including epigenetics (Jupe et al., 2019). As a result, these unintended effects also have to be assessed case by case and cannot be deduced from other 'events'. Moreover, experience gained from conventional breeding cannot simply be extrapolated to the risk assessment of GE organisms.

According to scientific literature (Adamczyk & Meredith, 2004; Adamczyk et al., 2009; Chen et al., 2005; Dong & Li, 2006; Huang et al., 2014; Luo et al., 2008; Nguyen & Jehle, 2007; Then & Lorch, 2008; Trtikova et al., 2015), the expression of Bt genes can, for example, be impacted by:

> the genetic or epigenetic background (the specifics of the plant genome and its gene regulation) of the varieties into which the additional genes are inserted;
> interaction with environmental factors and stressors; and
> the different stages of vegetation, i.e. growing, flowering, ripening.

Furthermore, the number of gene constructs, the location of insertion and their interaction with the plant genome can cause unintended changes in plant composition and phenotypical characteristics, such as seed dormancy, number of pollen and seeds, pollen viability and response to biotic or abiotic stressors.

Unintended effects caused by the insertion of the additional genes may, for example, trigger unexpectedly high or low levels of Bt toxins in the various plant tissues with substantial consequences for the environment and food production. A level that is too low can cause the plants to be insufficiently protected against infestations of the larvae of *Maruca vitrata*. Further, under these conditions, the insect pests can evolve more rapidly to become resistant to the expressed Bt toxin. A high concentration of Bt toxins, on the other hand, is relevant for assessing risks to non-target organisms and food safety.

Moreover, if the expression of the transgenes is substantially impacted by environmental conditions or interactions within the genome, this can cause a wide range of unintended and unpredictable effects on plant metabolism and their response to environmental conditions. Under such circumstances, risk assessment can hardly result in a favourable outcome.

In general, unintended changes affecting plant composition, metabolism and signalling pathways or response to environmental conditions can affect agro-ecosystems, food production and food safety as well as biodiversity and organisms that carry out essential ecosystem services, e.g. pollinators or associated soil organisms. Examples of potential adverse effects include higher vulnerability to stressors (such as diseases or pests) or enhanced gene flow and invasive spread into the environment. Unintended effects caused by the insertion of the additional genes may also trigger unexpectedly high or low levels of Bt toxins in plant tissues, with severe consequences for the environment and food production. It should be taken into account that the composition of cowpea including proteins, anti-nutritional compounds and biologically active compounds is complex, including potential allergens.

Therefore, the applicant has to make data available on the insertion of the genes, gene expression, the concentration of the additional proteins in the various plant tissues, as well as the impact of the gene insertions on plant gene activity, plant composition and biological characteristics.

Furthermore, data should be made available on the response of the plants to specific abiotic or biotic stressors that are known to occur in the region, or which may emerge due to climate change.

Gene flow to other cultivated varieties or wild relatives

West Africa is among the regions in the world with the earliest evidence of cowpea (*Finamore unguiculata*) cultivation, dating back more than 1500 years (D'Andrea et al., 2007). Therefore, cowpea is considered to be a important pan-African heritage that is closely related to the history of countries such as Ghana, South Africa and Nigeria.

Cowpea is morphologically variable and adapted to different environments, resulting in a wide range of local varieties (OECD, 2015). The nutritional composition of cowpea is impacted by genetic characteristics, agro-climatic conditions, biotic stresses and postharvest management (OECD, 2018). Interestingly, despite the considerable morphological diversity, limited genetic diversity occurs among cultivated cowpea varieties owing to a single domestication event that has given rise to all cultivated varieties (OECD, 2018). However, publications show that there are genetic differences depending on the region of cultivation that can be used for improving cowpea varieties by traditional breeding (Boukar et al., 2019; Chen et al., 2017; Fatokun et al., 2018; Ghalmi et al., 2010; Ogunkanmi et al., 2008; Oyatomi et al., 2016; Xiong et al., 2016; Zannou et al., 2008).

The *Vigna unguiculata* species complex is currently divided into eleven subspecies. Ten of the subspecies are perennial and one subspecies is annual (OECD, 2015). There are no apparent barriers to hybridisation or recombination between members of the different cultivar groups or with the wild cowpeas (*var. spontanea*) in the subspecies *unguiculata* (OECD, 2015). As stated in OECD (2015): *"The overall message is that crosses appear possible among all members of the Vigna unguiculata complex, but they vary from being easy to being difficult."*

Wild relative subspecies belonging to the group *Vigna unguiculata var. spontanea* are known to occur in Nigeria as well as in many other West African countries. *Vigna unguiculata var. spontanea* can be found mostly in disturbed areas (such as fields, field margins, roadsides and fallows) but also in natural ecosystems, such as those observed in Cameroon, Uganda and Ethiopia (OECD, 2016).

Gene flow between wild subspecies has been observed; its frequency is largely dependent on the subspecies, the size and location of the area of cultivation and the occurrence of insects. While often considered a rare event, according to a research project in Western Africa published in 2012, there were findings of gene flow between cultivated and wild relatives in each of the populations investigated (Kouam et al., 2012). As OECD (2016) summarises, cultivated cowpea readily crosses with wild cowpea in the same subspecies (i.e. *var. spontanea*) and can be crossed with members of the other subspecies of *Vigna unguiculata* but with varying degrees of difficulty.

There are several pollinating insects involved in the distances and success rates of gene flow but in many cases, their specific role still needs to be investigated. As stated in OECD (2015): „*Cross-pollination is usually less than 1%, but will vary somewhat with the cultivar and, more particularly, with the population of some insects. In several cases, the pollinators are not known, but honeybees (Apis mellifera) have been observed around cowpea flowers and thus have been implicated in pollination (…) In coastal Kenya and Burkina Faso, several large carpenter bee species (Xylocopa spp.) and leafcutter bee species (Megachilidae spp.) were considered potential cross-pollinators of cowpea (…), and it was shown that these same leafcutters and carpenter bees were the likely pollinators of the wild progenitor of cowpea (…) Casual observations made in California, Texas and Nigeria indicate that large bumblebees (Bombus spp.) may be responsible for the cross-pollination that occurs in cowpeas in these regions.*"

Under these conditions, gene flow will occur in the fields when the GE cowpea is grown in close vicinity to regional varieties and without sufficient distance to wild relatives in other bio-geographical zones. For example, as NEPAD's African Biosafety Network of Expertise (NEPAD-ABNE) explains on their website, 90 % of the pollination in cowpea stems from self-fertilisation[1]. In consequence, there is still 10 % cross-pollination.

Therefore, agricultural cultivation of the Bt cowpea is likely to cause the introduction of artificial genes into regional varieties, whether intentionally or unintentionally. Gene flow to wild relatives is also likely to occur in the longer term. Due to gene flow, the transgenes will be introduced into plants with largely heterogeneous genetic and epigenetic backgrounds. This finding is supported by Huesing et al. (2011) and reflects the perspective of experts affiliated with the biotech industry. In regard to gene flow of Bt cowpea, these experts state: *"Based on existing information, the panel determined that hybridization is likely to occur."*

In some regions, hybrid crossings of cultivated varieties and wild subspecies are even grown deliberately as fodder plants (OECD, 2016). Often wild cowpea plants are not uprooted from the field and appear to persist in the agroecosystem. The hybrid progenies may even end up being used by farmers for sowing and may be considered as fodder landraces (OECD, 2016).

This poses substantial risks for the protection of natural biodiversity which might become contaminated by pertinent gene flow from Bt cowpea. This risk is acknowledged by the International Institute of Tropical Agriculture (IITA) in its conservation strategy for genetic resources of cowpea and its wild relatives (IITA, 2010) which proposed urgent measures especially for Nigeria: *"Collecting missions should take place in the following four high priority regions: (…) Nigeria for wild cowpea (mainly subsp. unguiculata var. spontanea), as it is underrepresented in ex situ collections. Moreover, there is a risk of genetic contamination from the introduction of Bt-cowpea in the country (field trials started in 2009)."*

In regard to environmental risk assessment, effects on hybrid offspring and the effects on the next generation, considering regional varieties as well as wild relatives and their interactions with the environment, are relevant (for an overview of next generation effects see Bauer-Panskus et al., 2020). Crucial risks include the effects on

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1   http://nepad-abne.net/biotechnology/gm-crops-under-research-in-africa/cowpea/

the following: genetic stability, gene expression, gene function, pleiotropic effects, persistence, and invasiveness. These biological mechanisms are known to be impacted by the genetic or epigenetic background of the genome into which the additional genes are introduced by gene flow as well as by interactions with the environment. There is evidence that the biological characteristics of the offspring generation in many cases cannot be predicted from the original genetically engineered event (Bollinedi et al., 2017; Cao et al., 2009; Kawata et al., 2009; Lu & Yang, 2009; Vacher et al., 2004; Yang et al., 2017). If the offspring can persist and propagate in the environment, interactions with the environment or changes in the environmental conditions can, in addition, play a major role in triggering unintended biological effects (Fang et al., 2018; Matthews et al., 2005; Meyer et al., 1992; Then & Lorch, 2008; Trtikova et al., 2015; Zeller et al., 2010; Zhu et al., 2018).

Relevant risks posed to the environment are, for example, changes in the plant composition of the hybrid offspring. This can involve their metabolism and signalling pathways in such a way that essential ecosystem services performed by pollinators or associated soil organisms can be impacted. Other examples of potential adverse effects include the higher vulnerability of the plants' offspring to stressors, or enhanced gene flow and spread into the environment. Furthermore, effects observed in hybrid offspring are unexpectedly high or low levels of Bt toxins produced in the various tissues of the plants (see above).

It should be taken into account that if Bt toxins are produced in wild relatives of the cultivated cowpea, this is likely to confer higher fitness compared to wild-types, which can eventually result in replacement and displacement of the native species and have far-reaching disruptive consequences for associated ecosystems. According to NEPAD-ABNE, wild cowpea has to be considered to be an invasive and allelopathic species with much lengthier seed dormancy in comparison to cultivated varieties. This underlines the risk of Bt cowpea becoming invasive and replacing and displacing natural populations, thereby becoming disruptive to ecosystems beyond the fields. This is especially relevant since West Africa is a centre of the biological diversity of cowpea.

The example of Bt cotton in Mexico underlines the need for the precautionary principle to be applied in regard to GE plants with the potential to persist and propagate in the environment. A detailed investigation (Vázquez-Barrios et al., 2021) shows that there are disturbances in the interactions between the transgenic offspring of Bt cotton and their environment. The plants exhibit characteristics of invasive plants such as changes in their defence mechanism to herbivores which could not be predicted based on the intended trait. These findings have serious implications for the protection of wild cotton species because Mexico is one of the centres of origin of cotton.

Therefore, the risk assessment should take into account the presence and occurrence of regional varieties and wild relatives, their biological characteristics and their genetic differences compared to the variety used for field trials and cultivation. To obtain reliable data, experimental crossings should be conducted under contained conditions, such as in a closed greenhouse, before any field trials take place or cultivation can be considered.

Impact on non-target organisms

There are many publications showing that Bt toxins have several modes of action (for an overview see: Hilbeck & Otto, 2015; Vachon et al., 2012) that impact a broader range of non-target organisms than previously thought, and which in addition do not follow the boundaries of taxonomy (Hilbeck & Otto, 2015; van Frankenhuyzen, 2013). Based on research with the original bacteria *B. thuringiensis*, it was believed that, for example, Bt toxins such as Cry1Ab were only active against specific orders of insects, such as Lepidoptera. However, van Frankenhuyzen (2013) reports adverse effects beyond its presumed target species for 69 different insect taxa.

In general, selectivity and efficacy of Bt toxins can be influenced by many co-factors (Hilbeck & Otto, 2015; Then, 2010; Testbiotech 2021). In the case of cowpea, plant compounds, such as PI, naturally produced in the cowpeas, can cause Bt toxins to degrade much more slowly than in isolation. This can result in higher toxicity of Bt toxins if taken up together with the plant tissue when compared to the toxin in isolation (Zhang et al., 2000; Zhu et al., 2007; Pardo Lopez et al., 2009; Ma et al., 2013; Mesén-Porras et al., 2020). Indeed, there is evidence that toxicity of Bt toxins is increased if combined with PI such as those produced by cowpeas (MacIntosh et al., 1990; Zhao et al., 1999; Fan et al., 1999; Gujar et al., 2004; Cui et al., 2011).

It is known from scientific publications that co-factors which enhance the toxicity of Bt proteins can also impact their selectivity (for an overview see Then 2010): if synergistic or additive effects occur which increase the efficacy of Bt toxins, its selectivity may be decreased and a wider range of non-target organisms may become susceptible. Indeed some of the publications also indicate the effects of PI combined with Bt toxins on non-target insects. which will need more research (Babendreier et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Han et al., 2010).

Beyond that, there are several modes of action under discussion for Bt proteins. As Hilbeck and Otto (2015), Vachon et al. (2012), Latham et al. (2017) and Then (2010) show, there are complex modes of action which have to be taken into account.

These findings need to be carefully considered. They show that a detailed risk assessment is needed to address potential adverse effects on non-target organisms. As a result, due to the complexity as outlined, risk assessment of Bt cowpea cannot be concluded by testing the protein in isolation, or from data stemming from other events, or other plant species.

Pest replacement also has to be considered in this context: while *Maruca vitrata* abundance might be reduced, other pest insects can show higher rates of infestation. Such effects are already known to occur in Bt maize, Bt soybean and Bt cotton (Catangui & Berg, 2006; Catarino et al., 2019; Dorhaut & Rice, 2010; Kranthi & Stones, 2020; Li et al., 2020; Lu et al., 2010; Tabashnik et al., 2013; Wang et al., 2008; Zeilinger et al., 2016) and are likely to occur in cowpea since it is known that cowpea production is impacted by a wide range of additional insects which are not affected by this Bt toxin, including aphids, thrips and pod sucking bugs (Addae et al., 2020).

The OECD (2015) provides a list of non-pest arthropods associated with cowpea, including many pollinating and beneficial species that are natural enemies of cowpea pests and general predators (Table 8). In regard to the food web, it has to be taken into account that Bt toxins might accumulate in higher concentrations within the tiers of the food web, especially if taken up by predatory insects, e.g. beneficial predator wasps, feeding on the larvae of *Maruca vitrata* (Obrist et al., 2006 a/b; Paula & Andow, 2016; Zhang et al., 2006; Zhou et al., 2014). An impressive list of parasitoids and entomoviruses that attack the pod borer, *Maruca vitrata*, in West Africa can be found in OECD (2015, Table 7). As stated in OECD (2015), in addition to parasitoids, generalist predators also feed on cowpea insect pests. These include mites, beetles, ants, bugs and spiders.

Therefore, a list of organisms in Nigeria that could potentially be exposed to the plant material directly or indirectly should have been provided before any field trials were authorised or cultivation was allowed. These organisms should be subjected to specific tests in the laboratory or a greenhouse to provide the relevant data on the toxicity and impact of the plant material. The investigations should include synergistic effects of Bt toxins and PI as described and evidenced (MacIntosh et al., 1990; Zhao et al., 1999; Fan et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo-López et al., 2009; Cui et al., 2011; Ma et al., 2013; Mesén-Porras et al., 2020; see also Schlüter et al., 2020).

If the risks are not fully investigated, plant material from the Bt cowpea may have unexpected and severely adverse effects on ecosystem services, the food web and biodiversity if it is taken up by insects or wild species, e.g. birds or other non-target organisms.

Taking into account the rich biodiversity in Nigeria as well as the current threats to its conservation (Federal Republic of Nigeria, 2015), these questions are highly relevant to GE cowpea environmental risk assessment. Nigeria has several bio-geographical zones (Federal Republic of Nigeria, 2015) which need to be taken into account; there are substantial differences between these zones regarding fauna and flora. *"Each of these ecosystems has its own unique characteristics of wild fauna, higher and lower floral species and a huge collection of marine and freshwater aquatic species. In species diversity and endemism, Nigeria is highly endowed."*

There is already a substantial threat to biodiversity (Federal Republic of Nigeria, 2015): *"However, overall, biodiversity in Nigeria is highly threatened due to land use changes from agriculture and overgrazing, over exploitation of natural resources through extractive actors, invasive species and environmental pollution. According to the IUCN Red list 2013, Nigeria has a total of 309 threatened species in the following taxonomic categories: Mammals (26), Birds (19), Reptiles (8), Amphibians (13), Fishes (60), Molluscs (1), other Invertebrates (14) and Plants (168)."* There are reports of 20.000 insect species, including more than 1000 butterfly species, in the 'Cross-River-Nationalpark' (CBD, 2001; Terborgh, 2002).

The risks for biodiversity are especially relevant since there is considerable overlap between the centres of biodiversity in Nigeria, especially in its northern and central regions where cowpea is cultivated (FAO, 2004). In addition, small-scale farmers, in particular, might cultivate Bt cowpea in the highly diverse regions where gene flow is likely to expose ecosystems beyond the fields to Bt toxins.

Impact on microbial communities

Cowpea has unique symbiotic relations with specific communities of soil microorganisms (*rhizobia, mycorrhizae*) that enhance the flow of reduced nitrogen and phosphate into the cropping system (OECD, 2015). Bt cowpea produces Bt toxin in the roots and therefore the impact on soil organisms in the rhizosphere has to be further investigated.

It is also known that cowpea (like many plant species) are symbionts to specific endophytes (see, for example, Marzan et al., 2018). Endophytes and plants often engage in mutualism, with endophytes primarily aiding in the health and survival of the host plant, including issues such as pathogens and disease, water stress, heat stress, nutrient availability and poor soil quality, salinity, and herbivory (Hardoim et al., 2015). The diversity of the endophytic community varies with plant species, host genotype, type of tissue analysed, host age, climatic factors, and geographic distribution (see, for example, Farias et al., 2019). Therefore, if the genotype of the cowpea is changed and Bt toxins are expressed in all tissues of the plant, the composition of endophytes might also change. Consequently, plant response to the environment might also be altered. Given the crucial role of endophytes for plant health, this may have a detrimental effect on the health of the plants under specific environmental conditions.

As field trial publications on other Bt crops show, changes in the microbial community can be expected (Chen et al., 2011; Chen et al., 2012; Chen et al., 2017; Shu et al., 2017; Singh et al., 2013; da Silva et al., 2016; van Wyk et al., 2017). Especially relevant in this context are experiments showing a delay in degradation of Bt toxins in the soil if combined with cowpea trypsin inhibitor (Chen et al., 2012: see also Schlüter et al., 2020). The presence of the cowpea PI significantly delayed the degradation of Bt proteins produced by Bt cotton. Under these conditions, the repeated cultivation of transgenic cotton had considerable negative effects on the microbial properties and enzymatic activities in rhizosphere soil compared to those in the rhizosphere soil of non-transgenic cotton.

Health risks at the stage of consumption

As mentioned above, there are many publications showing that Bt toxins have several modes of action which impact a broad range of non-target organisms, irrespective of the boundaries of taxonomy. This is also the case with Cry1Ab. Furthermore, selectivity and efficacy can be influenced by co-factors and changes in the structure of the Bt protein (see above). In the case of cowpea, plant compounds, such as PI, can cause Bt proteins to show higher toxicity than it would in isolation. Indeed, specific findings are showing an increase in the toxicity of Bt proteins if combined with PI present in cowpea (MacIntosh et al., 1990; Zhao et al., 1999; Fan et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo Lopez et al., 2009; Cui et al., 2011; Ma et al., 2013; Mesén-Porras et al., 2020). Such synergetic effects not only enhance the effectivity (toxicity) of Bt toxins but may also impact their selectivity (for an overview see Then, 2010; Testbiotech 2021).

Whatever the case, these synergistic effects will result in higher toxicity of Bt toxins if taken up together with the plant tissue when compared to the toxin in isolation (see above and also: Then & Bauer-Panskus, 2017). Therefore, it is evident that, for example, feeding studies using the isolated protein or feeding studies using other Bt staple food, such as maize or soybean, are not reliable when assessing the potential health effects of Bt cowpea at the stage of consumption. Whole plant feeding studies should instead be conducted with Bt cowpea.

In this context, there is also cause for concern that Bt toxins can trigger non-allergic immune responses, e.g. adjuvant effects (Finamore et al., 2008; González-González et al., 2015; Ibarra-Moreno et al., 2014; Jarillo-Luna et al., 2008; Guerrero et al., 2004; Guerrero et al., 2007; Legorreta-Herrera et al., 2010; Moreno-Fierros et al., 2000; Moreno-Fierros et al., 2013; Rubio-Infante et al., 2018; Rubio-Infante et al., 2016; Vázquez-Padrón et al., 1999) which might contribute to chronic diseases or enhance immune responses. It is largely acknowledged that more data are needed on adjuvant and other potential immune responses caused by Bt proteins (see, for example, Rubio-Infante, 2016; Santos-Vigil et al., 2018).

The synergistic effects described by MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Zhang et al. (2000) Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Cui et al. (2011), Ma et al. (2013), Mesén-Porras et al. (2020), causing higher toxicity of Bt toxins are also relevant to risk assessment in regard to the immune system: combination with PI is likely to be associated with a delay in the degradation of Bt toxins after consumption. This delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance chronic inflammation and allergies. These effects can depend on the interactions with the intestinal microbiome (Parenti et al., 2019; Testbiotech 2021).

There are additional and specific reasons why Bt toxins Cry1Ab expressed in the cowpea should be tested in detail in regard to potential immune responses: According to NEPAD-ABNE (2017) and OECD (2018) some proteins naturally produced in cowpea beans are regarded as potential allergens. The occurrence of

some allergies indeed has been identified, although these are still rare for cowpea: Research published in 2000 investigated serum from six patients allergic to cowpea 41 kDa and 55 kDa proteins and which were identified as cowpea allergens (Rao et al., 2000). These proteins were detected in subspecies of *Vigna unguiculata* grown in Asia (*Vigna sinensis*) which are discussed to have a common origin as the African subspecies (Xiong et al., 2016). Thus, combining these proteins with immune reactive Bt proteins might lead to the emergence of new allergies.

Furthermore, the composition of cowpea in regard to proteins, anti-nutritional compounds and biologically active compounds is complex. NEPAD-ABNE lists phytic acid, trypsin inhibitor, raffinose, stachyose, verbascuose, hemagglutin as the most relevant anti-nutrients. Similarly, OECD (2016) lists hemagglutinin, tannin, trypsin inhibitors, oxalate, phytate, polyphenols and oligosaccharides. Therefore, any changes in plant composition that might be caused by the insertion of the additional genes and their impact on food safety have to be thoroughly assessed.

In regard to cowpea, it has to be taken into account that its uptake as a main staple food in many regions might cause high exposure of humans to Bt toxins. Therefore, risk assessment should take into account the concentration of Bt toxins and their degradation throughout the relevant stages of processing of all parts of the plants (pods, beans and leaves) meant for human consumption. As far as human consumption is concerned, cowpea is mainly grown for grain (dry and fresh) and sometimes also for fresh pods and leaves (OECD, 2018; Ashinie et al., 2020). In general, the green and fresh edible parts of the plants (pods, leaves and beans) will undergo less processing compared to dried beans.

Also in this context, it has to be taken into account that there are several bio-geographical zones in Nigeria (Federal Republic of Nigeria, 2015), which show substantial differences not only in respect to fauna and flora but also in agricultural and cooking practices and huge ethnic diversity. For example, in Nigeria, the cooking time of cowpea is traditionally reduced by cooking it with a naturally-occurring alkaline rock-salt known as 'kanwa' (OECD, 2018). Furthermore, soaking the cowpea before cooking is also widely used to reduce cooking time. These traditional practices may impact the degradation or non-degradation of Bt proteins in the diet.

In light of MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Zhang et al. (2000), Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Cui et al. (2011), Ma et al. (2013) and Mesén-Porras et al. (2020), it has to be considered that in raw or less processed cowpea used for human consumption, the toxicity and potential immune effects of Bt proteins are likely to be significantly higher compared to heated beans since heat may partially deactivate PI as well as Bt toxins. This needs to be carefully addressed in risk assessment.

Consequently, any application for field trials or agricultural cultivation needs to be accompanied by data regarding food and feed safety, encompassing long-term (chronic) feeding studies with whole plant food and feed, also taking into account fresh material and food which was processed to a lesser extent compared to dried beans.

B. Can the data provided by the applicant be regarded as adequate and sufficient to assess the risks of release, cultivation and consumption of GE Bt cowpea?

It is a generally accepted principle that GE organisms (or Living Modified Organisms (LMOs) as referred to by the Cartagena Protocol on Biosafety, to which Nigeria is a Party), can only be released into the environment if an adequate risk assessment was performed beforehand.

To be considered adequate, risk assessment has to consider intended and unintended effects and should be sufficiently robust and conclusive. This is the only way to meet the requirements of the precautionary principle underlying the Convention on Biological Diversity and the Cartagena Protocol. Furthermore, the objective of risk assessment under the Protocol is to identify and evaluate the potential adverse effects of LMOs on the conservation and sustainable use of biological diversity, also taking into account risks to human health.

In the following section, the data provided by the applicant are compared to the hazards and risks identified in the chapter above.

Expression of the genes inserted

The data submitted by the applicant provide some information on gene expression in the plant. Table 6 (page 34) of the application (AATF, 2018) gives an overview of expression data of Bt proteins expressed in the genetic background of two cowpea varieties.

Overview of expression data of Bt proteins expressed in the genetic background of two cowpea varieties (Source: Table 6, page 34 of AATF, 2018).

Tissue Samples	Cry1Ab (µg/g FWT)[a]		NPTII (µg/g FWT)
	709A (IT86D-1010)	709A (IT97K-499-35)	709A (IT86D-1010)
Leaf	5.1 (1.9–8.1)	3.8 (1.5–7.4)	0.33 (0.21–0.45)
Flower	4.5 (3.2–5.2)	16.4 (5.6–22.8)	0.21 (0.21–0.21)
Pods	3.7 (2.9–4.0)	9.4 (5.7–17.9)	0.35 (0.31–0.41)
Green Cotyledon	2.2 (1.95–3.0)	4.1 (1.6–6.7)	0.16 (0.15–0.16)
Dry Seed	2.5 (2.3–2.8)	2.6 (2.3–2.9)	1.5 (1.3–1.6)
Pollen	0.12 (0.06–0.18)	ND[b]	0.22 (0.21–0.23)
Anther Wall	1.1 (0.98–1.2)	ND	0.30 (0.29–0.32)
Roots	0.25 (0.18–0.33)	ND	0.18 (0.06–0.30)

[a] Values represent the mean of replicate samples with the lowest and highest individual values shown in parentheses. Concentrations are uncorrected for extraction efficiency and expressed in µg protein per gram fresh weight tissue (FWT). [b] ND = Not determined.

These results show surprising effects in regard to (i) the strong impact of genetic backgrounds on gene expression; (ii) the high concentration of Bt content and (iii) the strong impact of environmental factors on gene expression. These findings were, however, not discussed by the applicant.

(i) The data from different varieties show the strong impact of genetic background on Bt gene expression

There are only five data sets reported from both of the two varieties (including leaf, flower, pods, green cotyledon and dry seed) that can be used for comparison of the expression of the Bt gene. No statistical analysis is available in public to compare these data sets. However, at least two of the data sets ('flower' and 'pods') point to substantial differences which are likely to be of statistical relevance because the data range does not overlap. A third data set (green cotyledon) is also likely to be substantially different with only a small overlap range in

data from the two varieties. As a result, the impact of the genetic background on the expression of the Bt gene results in Bt protein concentrations that are not equivalent but, instead, substantially different.

These findings indicate that Bt gene expression in the cowpea is not stable, but shows significant variations caused by the genetic background. However, no further data were made available on Bt gene expression in any other specific varieties, landraces or wild relatives.

Further, no data sets for comparison were made available for the expression of the nptII genes.

(ii) High Bt toxin concentration in some plant tissues

To our knowledge, the concentration measured in the flowers (22,8 μg/g FW) is the highest concentration of Cry1Ab toxin measured in any plant tissue of any transgenic plant so far. In this case, fresh weight (FW) was used as a reference. Higher figures would have been expected if dry weight (DW) had been used as a reference (as is the case in most market applications for the EU). As Table 7 (AATF, 2018) of the application shows, the applicant is well aware that Bt toxins concentration in Bt cowpeas is higher compared to the data reported in the literature.

The highest concentration is measured in the flowers, this means that especially non-target insects visiting the plants for pollination and feeding will be exposed to the toxin. However, the applicant did not present any specific data which could be used to assess these specific risks.

Whatever the case, because the data (see above) appears to show a lack of stability in gene expression combined with high concentration of Bt proteins in some plant tissues, further data would be needed examining more varieties and landraces as well as all plant tissues throughout the various stages of vegetation. Without such data, environmental risk assessment is not conclusive and safety cannot be demonstrated.

(iii) Bt gene expression data is likely to be impacted by environmental factors

As further data from the applicant show (AATF, 2018), the concentration of Bt toxins varies substantially during the stages of plant growth and maturation. The applicant gives some explanation stating: *"Cry1Ab accumulation largely parallels the accumulation of total protein during leaf development and maturation, a result that is consistent with the fact that Cry1Ab expression is driven by the promoter of a gene encoding a major protein constituent of photosynthetically active tissue (small subunit of Rubisco) promoter used to drive expression of Cry1Ab."*

In addition, the applicant correctly explains that the promoter which controls the expression of Bt proteins in the transgenic plants, (the *Arabidopsis Rubisco Small Subunit* promoter) is known to be influenced by signalling pathways involving abscisic acid and jasmonates in several plant species (Reinbothe et al., 1994). The phytohormone jasmonate and derivates thereof have important roles as signalling molecules in plant defence, particularly against insect herbivores (Glazebrook, 2005; Howe & Jander, 2008). If these signalling pathways interact with Bt gene expression, environmental factors such as biotic and abiotic stressors may substantially influence Bt gene expression.

In awareness of this problem, the applicant (AATF, 2018) states that *"there is little evidence available to indicate that under field conditions the Rubisco Small Subunit promoter is significantly affected by environmental factors."* However, the absence of conclusive evidence is not sufficient to avoid this problem: cowpea is cultivated in several bio-geographical zones in Nigeria. Some field trials were conducted between 2009-2015 (see AATF, 2018, Table 8), but there were no targeted 'stress tests' performed under defined environmental conditions followed by detailed analysis of the genome x environmental interactions (see for example Trtikova et al., 2015). Therefore, the existing evidence of the responses of the transgenic cowpeas to specific environmental conditions is very

limited. Without showing stability in gene expression under a broad range of environmental conditions, risk assessment cannot be regarded as sufficiently reliable and conclusive. On the contrary, based on the data presented, it has to be assumed that sufficient stability in gene expression is lacking. It is likely that the Bt content in the plants is extensively impacted by the genetic background as well as by environmental conditions.

In regard to the assessment of plant composition and agronomic characteristics, no conclusions can be presented here. The applicant did not report any relevant differences between the data from the GE cowpea compared to the conventional plants. However, we did not have access to the data and can not take any conclusion on the reliability of these findings. In general, the design of field trials, the environmental conditions, the agronomic practises and the choice of the comparators all may influence the outcome of the comparative assessment. Therefore, it is important that the design of the field trials sufficiently represents the conditions under which the plants may be cultivated.

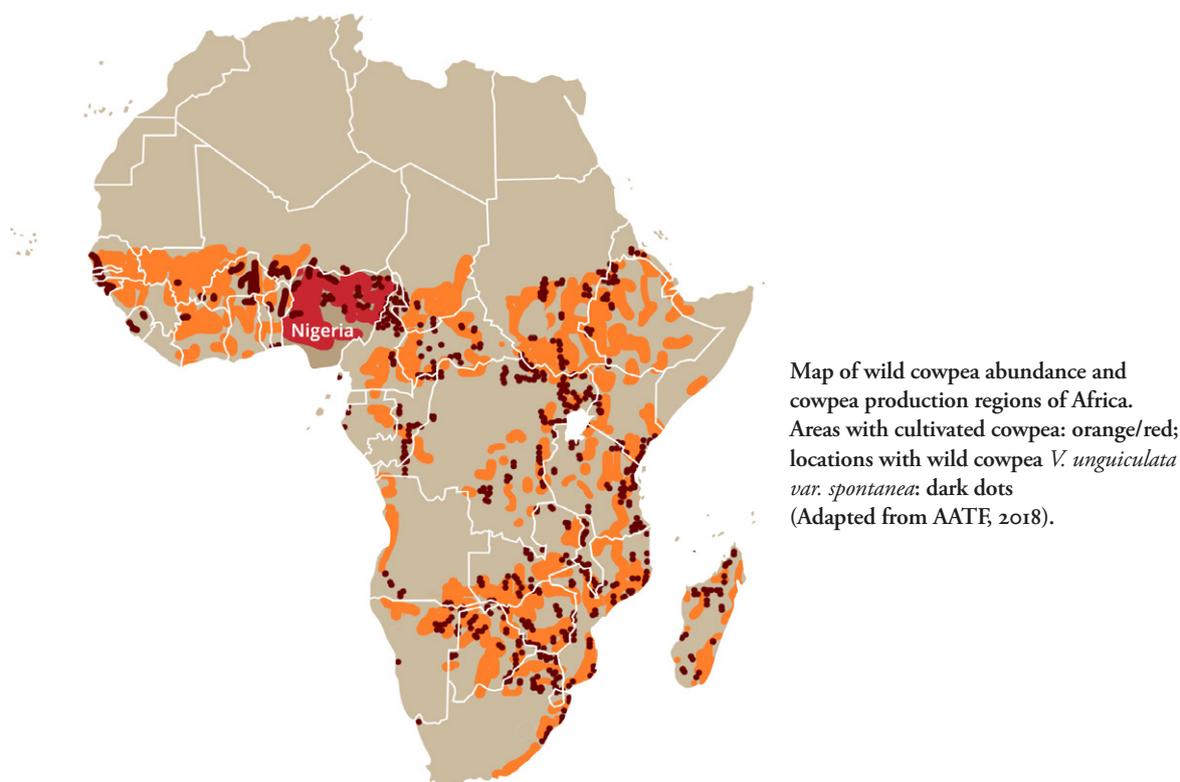### Initial conclusions on gene expression of the genes inserted

The data as provided indicate genetic instability in the expression of the additionally inserted genes. Therefore, further data would be needed on more varieties and landraces, including all plant tissues, throughout various stages of vegetation. These data should be produced under defined environmental conditions, e.g. in a climate chamber or greenhouse, to determine genome x environmental interactions. Without such data, environmental risk assessment is not conclusive, safety cannot be demonstrated.

Gene flow and next generation effects

It is known that there are barriers to hybridisation or recombination between members of the different cultivar groups or with wild cowpea (*var. spontanea*) in the subspecies *unguiculata* (OECD, 2015). However, these mechanisms can only limit but not prevent gene flow within and beyond the fields. As stated in OECD (2015): *"The overall message is that crosses appear possible among all members of the Vigna unguiculata complex, but they vary from being easy to being difficult."*

As mentioned, even Huesing et al. (2011), who represents the perspective of experts affiliated with the biotech industry, clearly state (in regard to gene flow of Bt cowpeas): *"Based on existing information, the panel determined that hybridization is likely to occur."*

As Figure 1 (page 19) of the dossier shows (AATF, 2018), wild relatives (*var. spontanea*) of domesticated cowpea grow in the same regions where cowpea is cultivated. Under these conditions, there is a high likelihood that viable hybrid offspring will occur not only in the fields but also in adjacent areas.

Map of wild cowpea abundance and cowpea production regions of Africa. Areas with cultivated cowpea: orange/red; locations with wild cowpea *V. unguiculata var. spontanea*: **dark dots (Adapted from AATF, 2018).**

The applicant states that *"the ecosystem into which AAT 709A could be disseminated is exactly the same as for conventional cowpea, namely managed agricultural environments where cowpea is being cultivated, and areas adjacent to managed agricultural environments."* Consequently, the exposure of traditional varieties, landraces, as well as wild relatives (*var. spontanea*) to gene flow from Bt cowpeas, will not be limited in spatial and temporal dimensions.

However, the applicant does not consider this to be a problem since reference is made to experiences from field trials (AATF, 2018):

"Apart from the presence of the insect resistance trait, the similarity of event AAT 709A to conventional cowpea based on extensive analysis including molecular characterization, protein expression, agronomic and phenotypic evaluation suggests that AAT 709A would not be expected to have any unintended fitness enhancing traits as a result of the modification. While it is possible that the insect resistance trait could confer a selective advantage to cowpea or wild cowpea that has acquired the trait under specific conditions of:

› *high insect pressure and*

› *presence of natural competitor plants that are significantly controlled by Maruca.*

As with conventional cowpea, it is highly unlikely that event 709A would adversely affect the environment through persistence or invasiveness."

However, the data derived from these field trials do not include any investigations of spontaneous crossings of the Bt cowpea with more heterogeneous backgrounds as would be the case if hybridisation with regional varieties or wild relatives occurs. No experimental crossings were performed with *Vigna unguiculata var. spontanea*. Instead, the applicant presented data on gene expression of the Bt cowpea *"prepared from progeny plants spanning ten selfing generations in direct line of descent from the original transformant"* (AATF, 2018). Further, the applicant reports on crossings of individual plants from two segregating generations in three genetic backgrounds only.

As shown in a review of existing publications (Bauer-Panskus et al., 2020), the biological characteristics of spontaneous hybrid offspring from transgenic plants cannot be predicted based on the data from the field trials with the original events. Therefore, hybrid crossings are needed to gather more data on the impact of the various genetic backgrounds on gene expression, also including crossings with *Vigna unguiculata var. spontanea*.

Otherwise, it cannot be excluded that crossings between the GE cowpea and other varieties or wild relatives can promote hybrid effects in ensuing generations, e.g. higher fitness and unexpected and non-predictable biological characteristics which may disturb or disrupt agro-ecological systems.

If the cut-off criteria proposed by Bauer-Panskus et al. (2020) for the assessment of *spatio-temporal controllability* had been applied in this case, it is most likely that the outcome would have shown sufficiently reliable risk assessment to be impossible. In this context, it has to be taken into account that Nigeria maintains the largest cowpea germplasm collection at the International Institute of Tropical Agriculture (IITA) with more than 15,000 landraces and over 2,000 wild relatives (Fatokun et al., 2018). Agricultural practices, such as re-sowing the harvest, exchanging seeds and also storage and transport of seeds, will allow the transgenic seed and its offspring to survive and persist in regional seed collections, landraces and potentially also in the collections of the IITA.

Therefore, claims by the applicant have to be rejected that there would be no threat of gene flow and potential spread of the Bt cowpea to biological diversity, traditional crops, farmers' varieties and sustainable agriculture. Such statements seem to ignore the importance of traditional seed saving for agro-biodiversity, seed bank collections, informed choice of breeders and farmers, food sovereignty and organic farming systems.

It seems that the applicant is not only ignoring risks to the environment but also willing to risk the common heritage of Nigerian farmers and breeders who have cultivated cowpea for thousands of years.

Initial conclusions on gene flow and next generation effects

Whatever the case, more data should have been provided by the applicant to assess the potential effects of hybridisation with regional varieties and wild relatives of cowpea. Based on the data provided by the applicant, releases of the Bt cowpea into the environment should not be allowed.

Further, the consequences of gene flow for seed collections, seed saving and traditional farming are not acknowledged by the applicant. Cultivation of the Bt cowpea will endanger the common heritage of African farmers.

Impact on non-target organisms

The applicant did not provide any data on relevant non-target organisms that are abundant in the regions where the Bt cowpea will be grown. This is surprising since Nigeria is the centre of origin for many endemic species and home to more than a thousand butterfly species belonging to the same group of insects (Lepidoptera) as the target species. Further, it is known that a large number of species interact with cowpea plants (OECD, 2015). However, no empirical data seem to be available regarding the susceptibility of these species listed by the OECD (2015).

As explained above, the selectivity and efficacy of the Bt toxins expressed in the plants can to a great extent be impacted and modified in comparison to natural sources of Bt toxins (Hilbeck & Schmidt, 2006; Then, 2010; Hilbeck & Otto, 2015; Latham et al., 2017). Relevant factors include the structure of the toxin as well as the combination with other stressors or plant components.

The data submitted by the applicant show that the Bt toxins expressed in the plants is not identical to Bt toxins found in natural soil bacteria. Instead, it was modified by Monsanto to improve expression and stability in the plant cell (Perlak et al., 1991). As described in the application (AATF, 2018), *"the nucleotide sequence of the cry1Ab gene was codon-optimized for plant expression and encodes a 615-amino acid protein (68.9 kDa) corresponding to the trypsin-resistant insecticidally active core protein following cleavage of the 1155-amino acid native Cry1Ab protoxin."*

In other words, the bacterial Bt pro-toxin which occurs naturally was shortened (truncated) so that the toxin in the plant is produced in its activated form. Therefore, the first step in the activation of the toxin, which takes place naturally in the intestine of the insects, is not needed for the Bt toxins produced in the plants. It is important to note that this activation is crucial for the specificity of Bt proteins (de Maagd et al., 2001). Therefore, changing the toxon´s structure might have altered its selectivity and efficacy as well, rendering it toxic for a broader range of non-target organisms (Hilbeck & Schmidt, 2006; Then, 2010; Hilbeck & Otto, 2015; Latham et al., 2017).

Besides the truncation, there are further changes in the structure of the Bt proteins produced in the plants (Perlak et al., 1991). It is not clear from the data presented by the applicant if this specific Bt protein is produced in other transgenic plants. For example, data published by Latham et al. (2017) does not result in any match of the gene construct if compared with other Bt plants.

Small changes in the structure of the protein can have a significant impact on its toxicity (see for example Pardo-López et al., 2009). Therefore, the statements of the applicant (AATF, 2018) generally assuming a *"narrow and specific toxicity"* only affecting *"interactions with susceptible Lepidopteran pests that feed upon the plant"*, without presenting specific experimental data regarding the Bt toxins as produced in the plants are not sufficiently substantiated.

Furthermore, the expression of Bt toxins in the plant tissue results in new paths of exposure and accumulation, including changes in quality and quantity when compared to the natural variants of Bt toxins (for an overview see Hilbeck et al., 2020). Beyond that, several modes of action of Bt proteins have been under discussion (Hilbeck & Schmidt, 2006; Then, 2010; Vachon et al., 2012; Hilbeck & Otto, 2015). As a result, a broader range of potentially affected organisms has to be taken into account than was originally assumed (see Frankenhuyzen, 2013 and Hilbeck et al., 2020).

However, the application (see also the diagram on page 53) only refers to one single mode of action. Further, the applicant ignores that the steps for activation of the toxin, as observed with natural toxins, are different to the ones produced in the Bt cowpea (see above). Consequently, the mode of action of Bt toxins presented by the applicant is not only outdated but has to be considered to be intentionally misleading:

"The spectrum of insecticidal activity of CryIAb is extremely narrow, with activity only against Lepidoptera (MacIntosh et al., 1990). The insecticidal specificity of CryIAb is the result of the numerous essential steps involved in producing an active protein toxin and its subsequent interaction with the epithelial cells in the insect midgut. To exert its insecticidal activity, CryIAb must:

1. *be ingested by the insect and solubilized in the insect gut,*

2. *be activated by specific proteolytic cleavage by insect midgut enzymes,*

3. *bind to specific receptors on the surface of the insect midgut, and*

4. *form ion channels in the gut membrane.*

The completion of all four of these processes results in damage to the insect midgut, leading to gut paralysis and death of the insect (Walters et al., 2010). The series of events leading to toxicity against Lepidopteran insects is highly specific to Lepidoptera, and does not occur in mammals, other vertebrates or other orders."

As the citation above shows, the applicant is aware of the findings of MacIntosh et al. (1990), who report much higher toxicity of Bt toxins if these are combined with PI produced in cowpea. It is astonishing that the reference is used to underline the selectivity of Bt toxins which is however not proven by MacIntosh et al. (1990). It is also remarkable that this publication was not integrated into the reference list of the application.

Whatever the case, the applicant, despite being aware of relevant publications showing specific risks, did not deliver any data to demonstrate the safety of the Bt cowpea in regard to non-target organisms. Instead, the applicant refers to publications reporting experiments with non-target organisms from other continents, not involving materials from the Bt cowpea (such as those mentioned in Huesing et al., 2011, or Koch et al., 2015). The applicant does not provide any studies with relevant Nigerian non-target species. Furthermore, there is no reference to existing experience with the cultivation of Bt crops in other countries, which the applicant considers to have demonstrated safety. However, as mentioned, experience with the cultivation of Bt plants belonging to other species that are grown in other regions and which have other gene constructs inserted, cannot be extrapolated to Bt cowpea cultivated in Nigeria. These findings are strongly supported by MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Gujar et al. (2004), Cui et al. (2011) who show the specific risks arising from Bt toxins being combined with PI produced in cowpea, causing much higher toxicity of Bt proteins through synergistic effects. Therefore, data from testing Bt toxins in isolation or from experiments with other plant species are of minor or no relevance for the risk assessment of the Bt cowpea.

It is plausible that the synergistic effects described MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Gujar et al. (2004), Cui et al. (2011) may also impact non-target organisms and the food webs. As mentioned, there are some publications indicating effects of PI combined with Bt toxins on non-target insects which will need more research (Babendreier et al., 2005; Liu et al., 2005a; Liu et al., 2005b, Han et al., 2010). However, no data are presented on the wide range of organisms that interact with the Bt cowpea, including insects, mites, nematodes and parasitic plants as well as birds, rodents and other mammals which feed on or interact with the plants. None of the relevant species (apart from *Maruca vitrata*) that are abundant in Nigeria was tested in any targeted study. There seems to be a general lack of empirical risk assessment studies. The most recent Bt cowpea study (Ba et al., 2018), which was compiled by experts who had already contributed to Huesing et al. (2011), does not mention any empirical testing of non-target organisms which are specific to Nigeria.

Initial conclusions on risks for non-target organisms

The applicant did not present any data on the susceptibility of non-target organisms that are abundant in Nigeria. The reasoning of the applicant as to why no such experiments are needed is based on the assumption that the *"spectrum of insecticidal activity of Cry1Ab is extremely narrow"* (AATF, 2018). However, this assumption is not based on sufficient scientific evidence. It does not acknowledge empirical findings showing toxicity of Bt toxins beyond the expected range of organisms. In addition, it ignores the existing scientific evidence on the factual complexity of the mode of action of Bt toxins.

Furthermore, the applicant did not take into account the existing evidence showing an increase in the toxicity of Bt proteins if these are combined with PI. Such synergistic effects not only enhance the effectivity of Bt toxins but may also impact their selectivity (Then, 2010). As a result, a much broader range of non-target organisms can be affected than could be expected from findings of testing Bt toxins in isolation (see also preliminary findings from Babendreier et al., 2005, Liu et al., 2005a, Liu et al., 2005b, Han et al., 2010).

Therefore, empirical testing of the relevant species is absolutely essential before concluding on the safety of Bt cowpea. The experiments should also take into account synergistic effects as shown or at least indicated by MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Zhang et al. (2000), Gujar et al. (2004), Liu et al. (2005a), Liu et al., (2005b), Zhu et al. (2007), Pardo-López et al. (2009), Han et al., 2010 Cui et al. (2011), Ma et al. (2013), Mesén-Porras et al. (2020).

Impact on microbial communities

The impact of Bt toxins on microbial communities is not adequately dealt with. As mentioned, cowpea has unique symbiotic relations with specific communities of soil microorganisms (rhizobia, mycorrhizae) (OECD, 2015). It is also known that specific endophytes are symbionts to cowpea (see, for example, Marzan et al., 2018). Furthermore, Bt toxins are also produced by the roots and, in addition, plant material such as that leftover from harvest will also contribute to additional Bt toxins in the soil.

However, this was given no consideration and no empirical tests were conducted by the applicant to assess changes in the associated endophytes or other communities within the plants' microbiome, or the soil rhizosphere. There is no data presented on the wide range of microorganisms that interact with the Bt cowpea, including symbionts (mycorrhizae, rhizobia, endophytes) or pathogens (bacterial, viral and fungal microorganisms).

Instead, the occurrence of soil bacteria naturally producing Bt toxins is used by the applicant to claim that a specific risk assessment would not be needed. While it is true that some soil bacteria do produce Bt toxins naturally (see, for example, Schnepf et al., 1998), these findings cannot in this case absolve the applicant from the responsibility of carrying out a detailed risk assessment. Compared to Bt toxins produced by soil bacteria, the Bt toxins produced in the cowpea differs extensively in structure and pattern of exposure to the environment (Hilbeck et al., 2020). Furthermore, Bt toxins naturally occurring in soil bacteria are not produced in combination with PI as is the case with the Bt cowpea.

Experiments showing a delay in degradation of Bt toxins in the soil if combined with cowpea trypsin inhibitor (Chen et al., 2012) are especially relevant in this context. The presence of the cowpea protease inhibitor delayed the degradation of Bt proteins produced by Bt cotton. Therefore, it can not be excluded that the repeated cultivation of Bt cowpea will have considerable negative effects on the microbial properties and enzymatic activities in rhizosphere soil.

Empirical testing of the relevant microbial species, including the plant microbiome and long-term impact on the soil rhizosphere, is necessary before concluding on the environmental safety of Bt cowpea. The experiments should also take into account synergistic effects as evidenced by MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Zhang et al. (2000), Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Cui et al. (2011), Ma et al. (2013), Mesén-Porras et al. (2020).

Initial conclusions on microbial communities

No empirical tests were conducted to assess changes in the relevant microbial communities. In the application, evidence of synergistic effects between PI (as naturally produced by plants) and Bt toxins was ignored. Based on existing data, it has to be expected that the degradation of Bt toxins in the soil may be significantly delayed in the presence of PI produced in the cowpea. Negative effects on the microbial properties and the enzymatic activities in rhizosphere soil can not be excluded after long-term cultivation. The environmental safety of Bt cowpea was not demonstrated since no data were provided by the applicant on the impact of Bt cowpea cultivation on the relevant microbial communities.

Food and feed safety

As mentioned, there are several publications showing that the toxicity of Bt toxins needs to be investigated in more detail, including in regard to human health at the stage of consumption (for an overview see: Then & Bauer-Panskus, 2017). The effects caused by the PI described by MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Zhang et al. (2000), Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Cui et al. (2011), Ma et al. (2013), Mesén-Porras et al. (2020) leading to higher toxicity of Bt toxins show that

the approach chosen by the applicant is inadequate: the applicant refers to mouse toxicity studies which aimed to assess short-term, high exposure to isolated Bt proteins. However, such studies are of little value since these experimental conditions are to a great extent different if synergistic effects between Bt toxins and plant material have to be expected, which is likely to lead to a much higher overall toxicity in the diet.

The synergistic effects between Bt proteins and PI, naturally produced in the cowpea not only enhance the efficacy of Bt toxins but may also impact their selectivity (Then, 2010). Therefore, a detailed investigation of potential health effects at the stage of consumption is absolutely essential and should take into account all traditional usages, including diets with raw cowpea.

The studies referred to by the applicant indicating a quick degradation of Bt toxins after ingestion are in contradiction to empirical findings: Chowdhury et al. (2003), as well as Walsh et al. (2011), have found that Cry1A proteins can frequently and successfully still be found in the colon of pigs at the end of digestion when they were fed with Bt maize. Thus, the Cry1A proteins can show much higher stability, at least in monogastric species, than predicted by digestion experiments with the protein in isolation. In this context, it is important to note that plant compounds present in cowpea, such as PI, can cause Bt toxins to degrade even more slowly than, for example, in maize. Indeed, the synergistic effects between the Bt proteins and PI are likely to be associated with a delay in the degradation of Bt toxins after consumption. Therefore, higher exposure to Bt toxins after consumption has to be expected if it is taken up together with the plant tissue, compared to the toxin in isolation.

The delay in degradation also causes higher Bt toxin exposure of the immune system via the intestinal tract. Under these conditions, immune responses, such as chronic inflammation and new allergies, may be triggered or enhanced. As shown above, there is substantiated concern that Bt toxins can trigger immune responses. Furthermore, it was shown that cowpea may produce potential allergens (Rao et al., 2000). Therefore, targeted studies should have been performed to exclude risks for the immune system. Instead, the applicant presents general statements such as "cowpea is not considered toxic, nor pathogenic to humans, and is not considered allergenic" (AATF, 2018), by referring to the AllergenOnline database. However, there is no discussion of the specific findings from Rao et al. (2000), also referenced by OECD (2018). No specific and experimental investigations were conducted by the applicant. Therefore, it can not be excluded that combining allergenic proteins with immune reactive Bt proteins might foster allergenic reactions via adjuvantic effects.

Further, the applicant does not mention the immunogenic properties of Bt proteins. Consequently, more detailed empirical investigations into the long-term impact of Bt cowpea at the stage of consumption would be needed before risk assessment can be concluded. However, the applicant did not even perform a single feeding study with the whole food and feed derived from the Bt cowpea.

Initial conclusions on health effects

The applicant does not acknowldege that cowpea is shown to produce potential allergens. In addition, it appears to be no awareness that Bt proteins are suspected of enhancing or provoking immune responses. Furthermore, the applicant is wrong in assuming that Bt proteins will be degraded quickly after consumption. The applicant is aware of relevant literature showing that synergistic effects causing higher toxicity of Bt toxins (MacIntosh et al., 1990), but does not mention these findings in the application.

Despite all the relevant findings, not a single feeding study with the whole food was performed. There are no experimental data on potential health impacts in humans or animals after ingestion of the cowpea. Consequently, the safety of the consumption of the Bt cowpea is not shown and health risk assessment cannot be concluded.

Conclusions and recommendations

The cultivation of the transgenic cowpea should be discontinued for the following reasons:

Molecular risk assessment

Existing data indicate instability in the expression of the additionally inserted genes. To assess their impact on environmental and health safety, more data would be needed on more varieties and landraces, including all plant tissues, throughout various stages of vegetation. These data should be generated under defined environmental conditions, such as in a climate chamber or greenhouse, to determine genome x environmental interactions. As long as these data are not made available, risk assessment is not conclusive, safety cannot be demonstrated.

Gene flow and next generation effects

Since gene flow to and hybridisation with regional varieties and wild relatives of the cowpea has to be expected, more data would be needed to assess the potential effects of hybridisation with regional varieties and wild relatives of cowpea. However, even if more data were made available, next generation effects emerging from spontaneous hybridisation will remain an area of major uncertainty and unknowns. Further, the consequences of gene flow for seed collections, seed saving and traditional farming are not acknowledged by the applicant. It is evident that the cultivation of the Bt cowpea in countries like Nigeria has a high potential to endanger the common heritage of African farmers. Nevertheless, it seems that no measures are foreseen to prevent gene flow from the Bt cowpea.

Non-target organisms

The applicant did not present any data on the susceptibility of non-target organisms particularly abundant in Nigeria. The assumption of the applicant that the *"spectrum of insecticidal activity of Cry1Ab is extremely narrow"* is not based on sufficient scientific evidence. It does not acknowledge empirical findings showing toxicity of Bt toxins beyond the expected range of organisms. The applicant also ignores relevant findings showing an increase in the toxicity of Bt proteins if combined with protease inhibitors (PI), naturally produced in the cowpea. Such synergistic effects not only enhance the efficacy of Bt toxins but may also impact their selectivity. Therefore, empirical testing of the relevant species, including the ecosystem services and the food web, is absolutely necessary before concluding on the safety of Bt cowpea for non-target organisms. The experiments should also take into account synergistic effects as evidenced by MacIntosh et al. (1990), Zhao et al. (1999), Fan et al. (1999), Zhang et al. (2000), Gujar et al. (2004), Liu et al. (2005a), Liu et al., 2005b, Zhu et al. (2007), Pardo-López et al. (2009), Han et al., 2010, Cui et al. (2011), Ma et al. (2013), Mesén-Porras et al. (2020). However, no such data were provided.

Microbial communities

No empirical tests were conducted by the applicant to assess changes in the relevant microbial communities. Synergistic effects between PI (as produced by plants) and Bt toxins were completely not acknowledged. However, there is evidence that the PI can prevent or delay normal degradation of Bt toxins in the soil and, after repeated cultivation, negative effects on the microbial properties and the enzymatic activities in rhizosphere may be expected. Since no data were provided by the applicant regarding the impact of Bt cowpea cultivation on relevant microbial communities, the environmental safety of the Bt cowpea was not demonstrated.

Health effects at the stage of consumption

The applicant does not acknowledge that cowpea was shown to produce potential allergens. In addition, the applicant seems to be unaware that Bt proteins are suspected of enhancing or provoking immune responses. Furthermore, the applicant is wrong to assume that Bt proteins will be degraded quickly after consumption. Despite mentioning the relevant literature, which shows that synergistic effects between Bt proteins and PI (MacIntosh et al., 1990; Zhao et al., 1999; Fan et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo Lopez et al., 2009; Cui et al., 2011; Ma et al., 2013; Mesén-Porras et al., 2020) may significantly enhance the toxicity of the diets, the applicant did not explain these findings in the application and did not provide any experimental data on real health impacts after ingestion of the beans. Not even one single feeding study with the whole food was performed. Consequently, the safety of the consumption of the Bt cowpea is not shown and health risk assessment cannot be concluded.

Based on the data available, the following worst-case scenario should be considered, in part or in combination, if the Bt cowpea is cultivated long-term and large-scale:

› decrease in soil fertility;

› increase in immune responses after consumption of cowpea;

› damage to the genepool of cowpea;

› damage to biodiversity involving non-target organisms and wild relatives of cowpea.

These scenarios and related adverse effects are all plausible, while the likelihood of their occurrence may be different from case to case. In any case, the risk assessment as conducted in Nigeria failed to adequately address these issues.

Based on the data available, we conclude that the identified risks are too serious and the uncertainties and unknowns in regard to risk assessment are so substantial that further release and cultivation of the Bt cowpea should not be allowed.

The reasons for concern as identified can also be relevant for other countries in which these GE cowpeas are approved or applied for release or marketing (such as Ghana).

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