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Comments on "Trial Summary on the Comparison of Various Non-Aflatoxigenic Strains of *Aspergillus flavus* on Mycotoxin Levels and Yield in Maize" by M.S. Molo, et al. Agron. J. 111:942–946 (2019)

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Dear Editor,

We read with great interest the recently published study in *Agronomy Journal* in which use of native *Aspergillus flavus* strains as biocontrol agents was described as an effective strategy to limit aflatoxin contamination of maize (Molo et al., 2019). The Molo et al. (2019) study was conducted during a single season, in a single field of a research station in the state of North Carolina. Yet, the authors, in our opinion, make nonrigorous comments and conclusions related to this important biocontrol technology.

We also have read online news articles—one in the web portal of the American Society of Agronomy—related to Molo et al. (2019) in which it is implied that the use of native atoxigenic strains to limit crop aflatoxin content is a novel concept (Chakravorty, 2019; Fisk, 2019). Merriam-Webster Dictionary defines novel as, "new and not resembling something formerly known or used". The concept of native atoxigenic strains to limit crop aflatoxin content was introduced in the late 1980s (Cole and Cotty, 1990; Cotty, 1989) and even discussed in a 2010 article (Burness Communications, 2010) by one web portal also providing commentary on Molo et al. (2019) (American Society of Agronomy, 2019).

Molo et al. (2019) and the online news commentaries on the article provide readers inaccurate interpretations of the status of aflatoxin biocontrol technology across the globe.

The article leaves the impression that use of native atoxigenic strains is new, whereas this concept is one of the bases of aflatoxin management through biocontrol with atoxigenic *A. flavus* active ingredients (Mehl et al., 2012) and use of native atoxigenic strains is mentioned in the title of a patent issued in 1992 by the US government (Cotty, 1992). The first reports of native atoxigenic strain efficacy in cotton (Cotty, 1990, 1994) and maize (Brown et al., 1991) were made almost three decades ago, and benefits of using native atoxigenic strains mentioned in the news commentaries in terms of soil and climate adaptation are concepts stressed in several publications (Cotty, 2006; Probst et al., 2011; Mehl et al., 2012; Atehnkeng et al., 2014, 2016; Bandyopadhyay et al., 2016). Results of Molo et al. (2019) should be considered as a preliminary indication that native atoxigenic strains are also beneficial for limiting maize aflatoxin content in North Carolina.

The first atoxigenic biocontrol product, *Aspergillus flavus* AF36, has been used for decades in areas where members of the vegetative compatibility group (VCG) YV36, to which the active ingredient fungus belong, are native (Cotty et al., 2007; Doster et al., 2014; Grubisha and Cotty, 2015). *Aspergillus flavus* AF36 was initially registered with USEPA for experimental commercial field treatment (up to 20,000 acres per year) in 1996 and received unrestricted registration from USEPA in 2003 (USEPA, 2003). Supporting data for registration of AF36 for use on cotton and subsequent amendments for use on maize, pistachio, almond, and fig (USEPA, 2012, 2017) included demonstration that the VCG is native to target areas and is effective in hundreds of farmer field trials of the various crops. The environmental safety of the product and its benefits to the farmers were additional criteria for registration (Cotty, 2006; Cotty et al., 2007; Doster et al., 2014; Ortega-Beltran et al., 2018). *Aspergillus flavus* AF36 is a commercially available product used annually by farmers to treat several hundred thousand acres in aflatoxin-prone areas of the United States, primarily in Arizona, Texas, and California. The active ingredient fungus is a strain native to the environments in which it is used. The range of YV36, apart from vast areas of the United States, extends over all areas of Mexico where frequencies of YV36 have been investigated (Ortega-Beltran et al., 2016). Indeed, because YV36 is native to Mexico, trials of AF36 in maize in Mexico are scheduled to take place during 2019 (N. Palacios, personal communication, 2019). The second atoxigenic strain-based biocontrol product, Afla-Guard, has an active ingredient that is distinct from AF36 but also native and widely distributed. Afla-Guard is commercially applied on maize and peanuts in the United States.

Core Ideas

- Native atoxigenic *Aspergillus flavus* have been used for decades to limit crop aflatoxin content.
- When applied correctly, commercial atoxigenic biocontrol products reduce aflatoxin content.
- Farmers continue using commercial products because treatments result in low aflatoxin levels.
- Influence of mating between atoxigenic strains in reducing aflatoxin content is questionable.
- Atoxigenic strains should be tested in multiple farmers' fields, in multiple environments, over multiple years.

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Abbreviations: VCG, vegetative compatibility group.

The online news commentaries reporting the so-called novel technology mention that, "commercial strains may not be the only answer' and that, "use of safe, native strains can be as effective, or more effective, than commercial strains" (Chakravorty, 2019; Fisk, 2019) despite one of Molo et al.'s core ideas: "Native and commercially available biocontrol strains are equally effective in reducing AF levels." Combinations of atoxigenic strains have not been used intensively in the United States, where use of single native-strain aflatoxin biocontrol products predominate (Cotty et al., 2008; Dorner, 2009; Mehl et al., 2012). However, an aflatoxin biocontrol product with four native strains for use in maize in Texas, FourSure (Shenge et al., 2017), has received a USEPA Experimental Use Permit (USEPA, 2016). The US Texas Corn Producers Board is seeking the registration of FourSure. Use of mixtures of native atoxigenic strains to treat almond, pistachio, and fig crops in California is also being pursued (Picot et al., 2018; Ortega-Beltran et al., 2019).

At some point, some or all of the strains reported by Molo et al. (2019) may be formulated into a product for use in commercially-produced crops. Reaching the commercial stage does not demote atoxigenic strains from native status if used in areas where they are common. Although no statistical data was provided, it seems that maize treated with AF36 and Afla-Guard had similar aflatoxin levels to maize treated with both IC6510+IC6511. In addition, Molo et al. (2019) does not contain information on how the strains were formulated and applied, the carrier (e.g., wheat, sorghum, barley), whether the commercial products Afla-Guard and AF36 were used, the dose, how physically separated were the treatments, whether strains applied in one treatment were found in maize from other treatments, or if the various tested strains differed in inducing kernel rot. The commercial products AF36 (current product is AF36 Prevail) and Afla-Guard have different formulations. It is important to point out that the strains were applied at tasseling. For maize, AF36 is recommended to be broadcasted 2 to 3 wk before tasseling. Moreover, the two commercial products were not tested in combination with one of the other tested strains. Further, data for effectiveness of IC6512, one of the two strains in the combination with the lowest aflatoxin content (although all treatments appear statistically the same), was not tested individually or the data is not provided.

In the news commentaries it is mentioned that, "using commercial strains can have some disadvantages. They usually need to be reapplied each year, at a cost of $20 per acre. Also, the application has to be done aerially or manually". It would be valuable to know how the strains were applied in the North Carolina study. Commercial biocontrol application is done once per cropping season. Frequently, as recommended by agronomists, susceptible crops are not planted on the same land in multiple years in succession but are rotated. Depending on the crop and the context, the biocontrol products (i.e., sterile grains coated with the active ingredient fungi) are applied aerially or by tractor as in most cottonseed and maize fields in the United States (http://www.azcotton.org/aflotoxin36/af36images/GroundApplicationPhotos/groundapp.html, accessed 5 Apr. 2019), by quad motorcycle in pistachio and almond orchards in the United States (http://www.azcotton.org/aflatoxin36/af36images/PistachioApplication/pistachioapp.html, http://kare.ucanr.edu/programs/Plant_Pathology/Biocontrol_of_Aflatoxins_in_Pistachio_and_Almond_Crops, accessed 5 Apr. 2019), or manually by smallholder maize and groundnut farmers in African nations (Bandyopadhyay et al., 2016). News commentaries (e.g., American Society of Agronomy, 2019) also indicate that the cost of an application, "can deter farmers from using commercial strains". Farmers treat crops with pesticides to protect against insects and plant pathogens. Agronomic packages including fertilizers and herbicides are far more expensive than the cost of treating crops with atoxigenic strains and the costs do not prevent input use. In the United States, there is a significant cost for growing a crop that exceeds maximum allowable aflatoxin levels. In aflatoxin-prone areas, treating crops with atoxigenic products provides opportunity to enter premium markets that in most cases would not be possible without biocontrol use.

It is also necessary to explain reasons to expect commercial atoxigenic strains will not remain in treated fields indefinitely, or for a long-term. Fungal community compositions are highly dynamic among and within years, even in single fields, and in both treated and non-treated areas (Bayman and Cotty, 1991; Mehl et al., 2012; Ortega-Beltran and Cotty, 2018). When fields are treated with atoxigenic strains, the composition of the fungal community associated with the crop changes so that the atoxigenic strains are very common. The changed community remains with crop remnants between seasons. However, other fungi resident in the soil also compete for resources associated with the next crop as do fungi arriving to the field from near and distant areas. Typically applied atoxigenic strains compose over 50% of the *A. flavus* community in the soil a year after application (Cotty, 2000, 2006) providing potential for additive effects with multiple year treatments and the potential to beneficially change the fungal community across large areas (Bandyopadhyay et al., 2016).

Molo et al. (2019) also indicates that combinations of biocontrol strains of opposite mating type may be more effective in limiting aflatoxin concentrations of treated crops compared to using single atoxigenic genotypes, which contain one of the two possible mating types in *A. flavus*. Occurrence of sexual recombination of *A. flavus* in sufficient frequency to be of epidemiological significance under natural conditions has been questioned in several well planned, multi-year studies examining fungal populations from cultivated and non-cultivated areas in the Americas and Africa (Grubisha and Cotty, 2010, 2015; Adhikari et al., 2016; Ortega-Beltran et al., 2016; Islam et al., 2018). On the other hand, if mating occurs under field conditions as a result of biocontrol applications as proposed by Molo et al. (2019), then atoxigenic strains composing commercial products would be mating with those of opposite mating type residing in the treated fields. The effect that Molo et al. (2019) claim to happen will also occur in those fields treated with a commercial product. However, in the Molo et al. (2019) paper it is stated, "The full impact of formulations comprising a mix of sexually compatible *MAT1–1*/*MAT1–2* mating types is expected long term because the mating process takes 6–11 mo in the laboratory (Horn et al., 2009a); however, a signature of genetic exchange and recombination has been detected just 3 mo after biocontrol application (M.S. Molo et al., unpublished data)." It is not clear how a long-term and fastidious process of sexual reproduction would impact the efficacy of strain combinations applied in the field in either the short term or the long term (Grubisha and Cotty, 2010, 2015). Some of the applied fungi will move beyond treated fields and likewise fungi from neighboring areas will migrate to treated fields during the course of the year. Conditions in any given field are by no means stable as in the laboratory. Also,

the signature of genetic exchange and recombination is reported to be long after strain application, 3 mo after harvest. Although not mentioned in the paper, the maize should have been harvested about 45 d after application. Rather than a recombination event influencing aflatoxin content, a more parsimonious explanation for the low aflatoxin content is simple competitive exclusion of aflatoxin producers residing in the field.

Use of single-genotype atoxigenic biocontrol products has allowed cultivation of susceptible crops in aflatoxin-prone areas. That is, use of single-genotype atoxigenic biocontrol products allows production of aflatoxin-compliant crops that can be commercialized in the most stringent markets across the globe. In the case of AF36, farmers in Arizona, Texas, and California continue to use AF36, a single-native atoxigenic strain biocontrol product because of the substantial reductions obtained when treating cottonseed, maize, and pistachio with the product. If AF36 was not effective in limiting crop aflatoxin content to compliant and safe levels, the thousands of farmers that use it would have discontinued treating their crops.

At this point we have only mentioned native atoxigenic biocontrol usage in the United States and early-stage usage in Mexico. Since 2003, the International Institute of Tropical Agriculture (IITA) in collaboration with the US Department of Agriculture–Agricultural Research Service (USDA–ARS), along with many partners, have developed several atoxigenic biocontrol products under the trade name Aflasafe for use in various African nations (www.aflasafe.com). Each Aflasafe product contains four atoxigenic *A. flavus* strains belonging to widely-distributed VCGs native to the countries for which the Aflasafe product was developed (Bandyopadhyay et al., 2016). Aflasafe strains composing different products contain partial or complete aflatoxin biosynthesis gene cluster deletions (Adhikari et al., 2016). Several Aflasafe products also contain active ingredient atoxigenic strains with opposite mating type (unpublished data, R. Bandyopadhyay et al., 2019).

Biocontrol products with multiple native atoxigenic strain active ingredients have been validated in African environments and approved by regulatory authorities responsible for pesticide registrations in several African nations (Bandyopadhyay et al., 2016; Schreurs et al., 2019) following field efficacy trials in hundreds of farmers' fields in multiple agro-ecological zones and during multiple years (Atehnkeng et al., 2008, 2014; Bandyopadhyay et al., 2016). Aflatoxin reductions in crops from treated fields range from 75 to 100% compared to untreated adjoining crops, even in highly challenging conditions that smallholder farmers frequently face across Africa. The reductions are observed both at harvest and even after poor storage (Atehnkeng et al., 2014). Aflasafe products are commercially available for use in Nigeria, Kenya, Senegal, The Gambia, Burkina Faso, Ghana, Tanzania, Zambia, Malawi, and Mozambique (Bandyopadhyay et al., 2016, 2019; Schreurs et al., 2019). More Aflasafe products are expected to be registered soon for use in other African nations.

Use of Aflasafe products, composed of mixtures of native atoxigenic strains, is also mentioned in a paper reviewing cultural and genetic approaches to limit crop aflatoxin content (Ojiambo et al., 2018). Carbone, a co-author of the Molo et al. (2019) paper, is also a co-author of that publication. In addition, development of biocontrol products using native atoxigenic strains is at different stages in Italy (Mauro et al., 2015, 2018), Argentina (Alaniz Zanon et al., 2016; Camiletti et al., 2018), China (Yin et al., 2009; Zhou et al., 2015), Iran (Houshyarfard et al., 2014), Thailand (Pitt et al., 2015), and other countries from where publications are not available (e.g., Romania, Serbia, Pakistan, Spain, and Costa Rica).

The observation made by Molo et al. (2019) regarding increased yield as a result of biocontrol application is quite relevant and we concur that it deserves additional research efforts. In the same vein, smallholder farmers have expressed that their groundnut crops produce higher yields when treated with Aflasafe SN01, a product developed for use in Senegal and The Gambia (https://aflasafe.com/2019/02/05/farmer-and-consumer-voices-on-aflasafe-sn01-beyond-beating-aflatoxin-in-food-in-the-gambia/, accessed 6 Apr. 2019). In Nigeria from 2013 to 2017, the thousands of maize farmers that used Aflasafe to treat over 60,000 ha of commercially-produced maize had on average a 50% yield increase compared to the usual maize yield in their respective areas (Schreurs et al., 2019). The yield increase was attributed to improved agronomic practices and correct utilization of fertilizers, insecticides, and fungicides, among other inputs. Influence of Aflasafe application on yield should also be investigated in Nigeria, The Gambia, and elsewhere.

In conclusion, the concept of using native atoxigenic strains has been in the public domain for decades and is not novel. The use of biocontrol products with multiple native atoxigenic strain active ingredients has been sought for well over a decade both in Africa and the United States. There was little to no opportunity for a recombination event to occur between atoxigenic strains of opposite mating type during the short period between application and harvest, and other mechanisms should have been responsible for the observed low aflatoxin levels. Testing of atoxigenic strains during research studies is not enough to obtain registration; commercial usage is possible after a mandatory, extensive registration process, development of infrastructure, technology transfer, and commercialization strategies that can span over 5 yr. Incidentally, an approval system accepting use of efficacy, ecotoxicological, and toxicological data from the diverse research efforts already undertaken during registration of current commercial products is needed to fast track the registration process of future products (Ortega-Beltran et al., 2019). Finally, field efficacy of atoxigenic strains should be conducted in multiple farmers' fields, in multiple agroecological zones, and over multiple years.

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A RESPONSE TO THE LETTER TO THE EDITOR FROM ORTEGA-BELTRAN AND BANDYOPADHYAY

Dear Editor,

The use of atoxigenic strains of *Aspergillus flavus* as biocontrol agents has proven to be very effective in reducing aflatoxin contamination in the continental United States (Cotty, 1990; Dorner, 2004, 2005) and worldwide (Atehnkeng et al., 2016; Bandyopadhyay et al., 2016; Camiletti et al., 2018; Mauro et al., 2018). The preceding commentary provides a very good overview of how native strains of *A. flavus* have been leveraged to reduce aflatoxin contamination of crops. As reported by the authors of the commentary, biocontrol using atoxigenic *A. flavus* strains is very effective not only in the short term of a single growing season but there is also some carry over to multiple years (Cotty, 2006; Pitt and Hocking, 2006; Yin et al., 2008). The Molo et al (2019) study takes this successful biocontrol strategy one step further by integrating our understanding of the population biology of *A. flavus* and specifically fungal mating in selecting strains for biocontrol products. The news article recognizes this novelty in the title, "Fungal mating: Next weapon against corn aflatoxin?" (Chakravorty, 2019, Fisk, 2019). As pointed out in the news article when referring to Molo et al. (2019): "Unexpectedly, the study also showed that certain combinations of native strains are more effective than commercial strains in reducing aflatoxin levels. That's because the combinations take advantage of fungal biology: their mating types are compatible, allowing them to reproduce and sustain their population." Fungal mating is the novel aspect of the Molo et al. (2019) paper, and not "native strains" per se or mixtures of native strains that do not consider mating type.

Throughout this commentary the authors refer to statements made in the news articles (Chakravorty, 2019; Fisk, 2019) which put more emphasis on the native strains than fungal mating. This is unfortunate because Molo et al. (2019) categorically states that, "Native and commercially available biocontrol strains are equally effective in reducing AF levels." and that "Deploying strains of opposite mating types in combination can lead to the greatest reduction in AF contamination". The authors of this commentary are clearly conflating their critique of the news article and of the Molo et al. (2019) paper. Molo et al. (2019) do not make misleading comments on biocontrol and its efficacy and state as a core idea that, "Biocontrol strains are effective at reducing AF levels in maize." The Molo et al. (2019) paper reports that a formulation comprising native strains of opposite mating types could be advantageous in improving the efficacy of existing single strain biocontrol formulations. The authors of the commentary are not acknowledging one of the main points of the Molo et al. (2019) paper: Native strains of opposite mating type can lead to greater reduction of aflatoxin contamination of maize. Molo et al. (2019) was published as a "Note and Unique Phenomena" and as such the findings are preliminary but yet compelling. Molo et al. (2019) for the first time show that the mating type composition of a biocontrol product may further reduce aflatoxin concentrations and possibly also increase yields.

Mating Type as a Biocontrol Selection Criterion

Up to now fungal mating and *A. flavus* evolutionary lineages as described by Molo et al. (2019) have not been criteria to consider in creating new biocontrol formulations. As pointed out in this commentary there are biocontrol formulations (e.g. Aflasafe) that comprise multiple *A. flavus* strains, but mating type and fungal evolutionary lineage were not a consideration in selecting those strains. Since the discovery of mating types (Ramirez-Prado et al., 2008) and the sexual state in *A. flavus* (Horn et al., 2009a), researchers have been able to type strains in existing biocontrol formulations. For example, the strains used in Afla-Guard and AF36 biocontrol products are of the same mating type (*MAT1-2*) but belong to different fungal evolutionary lineages (IB and IC, respectively) which might be important in the ecology of these organisms (Drott et al., 2017). In some cases, the mating types of strains used as active ingredients in other biocontrol products have been reported [e.g. AF-X1; Mauro et al. (2018)] or determined but not disclosed (e.g. Aflasafe) but detailed field trials on how different mating type combinations function in reducing aflatoxin contamination have not been conducted.

Mating Type as a Factor in Biocontrol

The general idea behind biocontrol is that you apply a competitive atoxigenic strain of *A. flavus* to the crop prior to harvest to reduce overall aflatoxin contamination in a field. When investigating the impact of commercial atoxigenic strains (Afla-Guard and AF36) as biocontrol agents on the genetic structure of *A. flavus* in field populations over time, we see in the short term (e.g. 45 d after biocontrol application) a strong signature of clonality and mating type distributions that are highly skewed to one mating type *(MAT1-2)*, which would be consistent with the competitive exclusion hypothesis (Cotty and Bayman, 1993; Mehl and Cotty, 2009; Mehl et al., 2012). After 3 mo, in addition to clonality, there is also a signature of genetic exchange between the applied biocontrol and native strains, which would further reduce aflatoxin levels in progeny strains (Olarte et al., 2012). After 1 yr, field populations re-establish mating type equilibrium and pre-biocontrol aflatoxin levels are restored (Molo, 2018). The detection of genetic admixture between the biocontrol strain and native strains suggests that populations can shift, even when the applied biocontrol agents are of a single mating type. Molo et al. (2019) show for the first time that the shift towards lower aflatoxin levels can be greater if biocontrol products include strains of compatible mating type. The Molo et al. (2019) paper is the first study to show that mating type composition and distribution in fields may have a role in biocontrol and sets the stage for further experiments to understand the underlying genetic mechanisms that can explain the role of sexual reproduction in the efficacy of biocontrol and give us insights into how to improve it.

Evidence for Sexual Reproduction in *A. flavus*

There is accumulating evidence that sexual reproduction within *A. flavus* is happening in nature and impacting the population genetic structure on recent time scales: (i) Sexual reproduction in as little as 8 wk and up to 6 mo (Horn et al., 2016, 2014). (ii) High turnover of vegetative compatibility groups (VCGs) in the same regions from year to year (Atehnkeng et al., 2014; Bayman and Cotty, 1991) and in progeny strains when compared to parental VCGs after a single generation of sex (Olarte et al., 2012). This is an important observation because each VCG is a clone and all isolates within a VCG are of the same mating type, either *MAT1-1* or *MAT1-2*. Therefore, new VCGs can only arise from mating between parental strains of different VCGs; there is little evidence of recombination within VCGs through parasexuality (Papa, 1973) in the field (Moore et al., 2009). (iii) Crossing over and independent assortment of *A. flavus* chromosomes in laboratory crosses (Horn et al., 2009a; Olarte et al., 2012, 2015) where the fertility of parental strains inferred from these crosses appears to be borne out under field conditions (Horn et al., 2016). This direct evidence is corroborated by numerous population genetics studies that go back to 1998 providing indirect evidence of recombination in field populations of *A. flavus* worldwide (Geiser et al., 1998, 2000; Moore et al., 2009, 2013, 2017; Ramirez-Prado et al., 2008) and among closely related aflatoxin-producing species (Carbone et al., 2007a, 2007b; Horn et al., 2009b, 2009c, 2011).

There are many drawbacks and limitations in using microsatellites as genetic markers in the studies reported in the commentary (Grubisha and Cotty, 2010, 2015; Islam et al., 2018; Ortega-Beltran et al., 2016) particularly when it comes to the analysis and interpretation of recombination (Putman and Carbone, 2014), as pointed out in a recent *A. flavus* population genetic study using the same set of microsatellite markers (Drott et al., 2019; Grubisha and Cotty, 2009). Again, this emphasizes the importance of examining single nucleotide polymorphisms from multiple loci (Geiser et al., 1998, 2000; Moore et al., 2009, 2013, 2017; Okoth et al., 2018; Taylor et al., 1999) and genome-wide (Molo, 2018) for determining patterns and rates of recombination in populations of *A. flavus*.

Considering all of the evidence, it is clear that recombination is the more parsimonious explanation than mutation alone for the observed genetic variation in *A. flavus*. The authors of this commentary cannot ignore the overwhelming direct and indirect evidence of the importance of sexual reproduction in shaping both historical and contemporary, including seasonal, genetic variation in *A. flavus* populations, and how this variation impacts population genetic structure and biocontrol. Not a single paper reporting on recombination and sexual reproduction in populations of *A. flavus*, which are relevant because they include Afla-Guard and AF36, has been cited in this commentary, and we note that the citation of Horn et al. (2009) was in reference to what was written in Molo et al. (2019). While the commentary is up-to-date with the current state-of-the-art of biocontrol using atoxigenic native strains it falls short in citing the breadth of work on the population genetics and mating biology of *A. flavus* which are paving the way to new technologies with the potential to greatly enhance current biocontrol practices.

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