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GENETICALLY MODIFIED CORN IN BRAZIL: HISTORICAL, RESULTS AND PERSPECTIVES

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Abstract – The objective of this work was to review the history of genetically modified (GM) corn in Brazil, and the results obtained since its introduction, as well as the perspectives for new technologies. GM corn was planted for the first time in Brazil in 2008 and, a few years later, it reached more than 80% of the planted area. Currently, the GM corn area in Brazil is close to 90%. The traits introduced in corn are related to herbicide tolerance and insect resistance, and the benefits for farmers and the environment in these 12 years were enormous. GM events also impacted plant breeding, and breeding methods needed to be adapted to include the introduction of GM events into germplasm. New emerging technologies, such as gene editing and synthetic biology, may have a new impact on corn improvement, creating new traits, many of them non-transgenic. These new technologies have the potential to improve traits associated with plant yield and tolerance to abiotic stresses.

Keywords: *Zea mays*, Herbicide tolerance, Insect resistance, Transgenic corn, Precision breeding technologies

MILHO GENETICAMENTE MODIFICADO NO BRASIL: HISTÓRICO, RESULTADOS E PERSPECTIVAS

Resumo - O objetivo deste trabalho foi revisar o histórico do milho geneticamente modificado (GM) no Brasil e os resultados obtidos desde sua introdução, bem como as perspectivas de novas tecnologias. O milho GM foi plantado pela primeira vez no Brasil em 2008 e, alguns anos depois, atingiu mais de 80% da área plantada. Atualmente, a área de milho GM no Brasil está próxima a 90%. As características introduzidas no milho estão relacionadas à tolerância a herbicidas e resistência a insetos, e os benefícios para os agricultores e o meio ambiente nesses 12 anos foram enormes. Os eventos GM também impactaram o melhoramento de plantas, e os métodos de melhoramento precisaram ser adaptados para incluir a introdução de eventos GM no germoplasma. Novas tecnologias emergentes, como edição de genes e biologia sintética, podem ter um novo impacto no melhoramento do milho, criando novas características, muitas delas não transgênicas. Essas novas tecnologias têm o potencial de melhorar as características associadas ao rendimento das plantas e à tolerância a estresses abióticos.

Palavras-chave: *Zea mays* , Tolerância a herbicidas, Resistencia a insetos, Milho transgênico, Tecnologias de melhoramento de precisão.

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GM Corn in Brazil

Genetically Modified Organisms (GMO) include any biological entity whose genetic material was modified using genetic engineering techniques, including the production and manipulation of recombinant DNA/RNA. The obtention of Genetically Modified (GM) crops are frequently pursued for improving current agronomical traits or introducing new traits that are naturally not found in a particular specie. Several sources of donors for non-related genetic material have been used for GM crop production, referred to as transgenes, including virus, microorganisms, animals, and other plants (Kumar et al., 2020).

Corn is an important food and feed crop around the world and has been a target crop for genetic modification since the first successful report using a protoplast transformation methodology (Rhodes et al., 1988). Since then, the transformation methodology evolved to become more efficient by introduction of particle bombardment procedure (Gordon-Kamm et al., 1990) and reliable after advances in *Agrobacterium*-mediated transformation (Ishida et al., 1996). Currently, several specific protocols for transformation of recalcitrant commercial corn inbred lines are available, including multiple selection systems (Yadava et al., 2017).

GM corn events were first commercialized in 1996 in the United States of America and contained traits providing herbicide tolerance and insect resistance. Since 1996, the global area of genetically modified crops has increased from 1.7 million hectare to 190.4 million hectares in 2019. Corn comprises 32% of GM crop area worldwide, with 60.9 million ha cultivated globally in 2019. GM corn also comprise 31% of all corn area in the world. The United States is the main producer of biotech crops with a planted area of 71.5 million hectares, followed by Brazil (52.8 million ha), Argentina (24 million ha), Canada (12.5 million ha) and India (11.9 million ha). In United States, 33 million hectares of GM corn was planted in 2019, an adoption rate of 92%. Currently, the US corn biotechnology market counts on corn traits including herbicide tolerance, insect resistance, drought tolerance, increased yield, male sterility, and fertility restoration, however stacked traits for combining insect resistance and herbicide tolerance are predominant (ISAAA Brief, 2019).

Despite the increasing adoption of biotech products in the United States fields, the first authorization for cultivation in Brazil occurred in 2007 by CTNBio – National Biosafety Technical Commission, and farmer adoption started from season 2008/2009. Although Brazil started later in GM corn cultivation, the area of GM corn in Brazil increased fast, from 1.8 million ha in 2008 to 16.6 million ha in 2019. Since 2013 the adoption of GM corn in Brazil is higher than 80%, and above 88% since 2016 (Figure 1).

CTNBio is the agency responsible for evaluating human, animal, and environmental aspects regarding GM crops in Brazil. Its role is defined by Brazilian Law No. 11.105 from March 24th, 2005, also known as the Biosafety

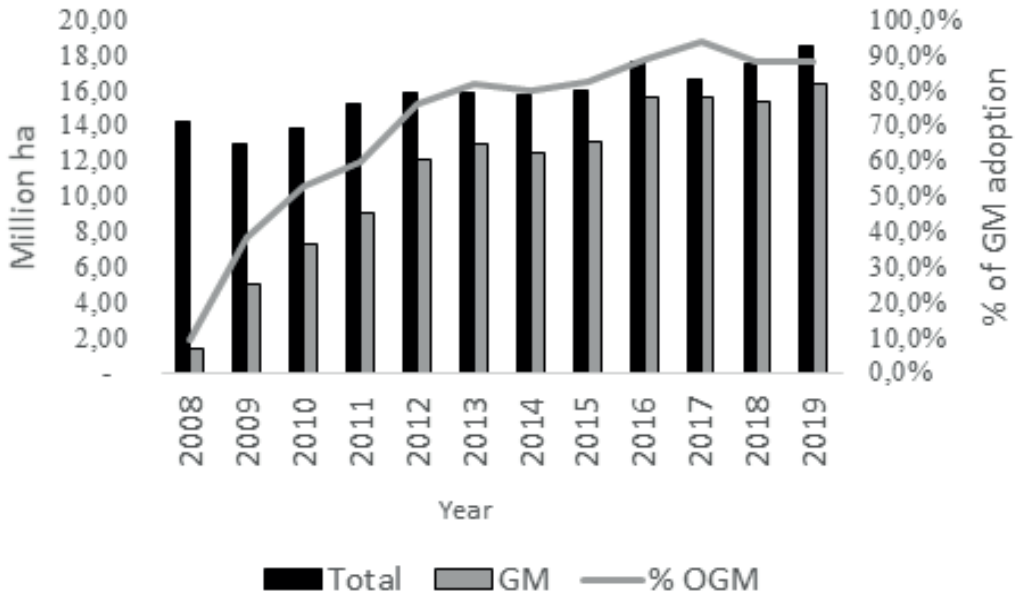

Figure 1. Evolution of GM corn in Brazil and rate of adoption in the first 12 years after launching. Adapted from ISAAA Briefs 2008 to 2019 (https://www.isaaa.org/default.asp) and CONAB historical series (https://www.conab.gov.br/info-agro/safras/serie-historica-das-safras?start=20).

Law. To date, CTNBio has been issued commercial clearance for corn, soybean, cotton, bean, eucalyptus, and sugarcane traits. Corn traits that received approval for cultivation are most composed by herbicide tolerance, insect resistance, and combinations of these traits.

For Brazilian planting season 2019/2020, 196 corn cultivars were available, from which 131 presented transgenic events and 65 were conventional cultivars. The technologies PowerCore™ Ultra, VT PRO3®, and VT PRO2® were predominant, representing 19%, 19%, and 12% of total cultivars containing biotechnologies. Biotechnology events can be found from one to four stacked events like in PowerCore™ Ultra, a combination of Herculex®, YieldGard VT Pro®, Roundup Ready™ 2, and Viptera™. VTPRO3® is a combination of YieldGard VT Pro® and Roundup Ready™ 2. Leptra® is a combination of Viptera™, YieldGard®, and Herculex®. Regarding the destination of corn cultivars for 2019/2020 season, 52% of cultivars were exclusively for grains, 45% for silage, and 3% special use (Pereira Filho and Borghi, 2020). The main technologies and combinations are described in Table 1 and Table 2.

Herbicide Tolerance Traits in Corn

Herbicide tolerance traits present in commercial approved GM corn for the Brazilian market confer plants tolerance to glyphosate, ammonium glufosinate, 2,4-D, dicamba, and combinations (Table 1). Currently, only glyphosate tolerant hybrids and ammonium

Table 1. Single trait Genetic Modified events approved for commercial use in Brazil.

Event Code	Commercial Trade Name	Proteins	Function	Approval Year
MON-ØØ6Ø3-6	Roundup Ready™ 2	CP4 EPSPS	Glyphosate Tolerance	2008
MON-ØØØ21-9	GA21®	mEPSPS	Glyphosate Tolerance	2008
ACS-ZMØØ3-2	Liberty Link®	PAT	Ammonium GlufosinateTolerance	2007
DAS-Ø15Ø7-1	Herculex®	Cry1Fa2 and PAT	Ammonium Glufosinate Tolerance and Insect Resistance	2008
MON-89Ø34-3	YieldGard VT Pro®	Cry2Ab2 and Cry1A.105	Insect Resistance	2009
SYN-IR162-4	Agrisure Viptera™	VIP3Aa20	Insect Resistance	2009
MON-88Ø17-3	YieldGard VT Rootworm® RR2	CP4 EPSPS and Cry3Bb1	Glyphosate Tolerance and Insect Resistance	2010
MON-ØØ81Ø-6	YieldGard®	Cry1Ab and CP4 EPSPS	Glyphosate Tolerance and Insect Resistance	2007
SYN-BTØ11-1	Agrisure®	Cry1Ab and PAT	Ammonium Glufosinate Tolerance and Insect Resistance	2007
DAS-4Ø278-9	Enlist® Corn	AAD-1	2,4-D Tolerance	2015
MON-87411-9	n/a	Cry3Bb1, dvsnf7*, CP4 EPSPS	Glyphosate Tolerance and Insect Resistance	2016
MON 95379	n/a	Cry1Da_7, Cry1B.868	Insect Resistance	2020

Source: CTNBio - http://ctnbio.mctic.gov.br/
*double-stranded RNA transcript

glufosinate tolerant hybrids are being used in commercial hybrids.

Glyphosate (N-phosphonomethyl-glycine) is a non-selective, systemic, post-emergence herbicide that specifically binds to and inactivates the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is part of plant biochemical shikimate pathway. The shikimate pathway is involved in the biosynthesis of the aromatic amino acids tyrosine, phenylalanine and tryptophan, as well as other aromatic compounds. When conventional plants are treated with glyphosate, the production of aromatic amino acids is compromised, which

Table 2. Description of stacked commercial traits used in Brazil.

Commercial name	Events	Trait	Proteins	Approval Year
PowerCore™	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	HT and IR	Cry1A105, Cry2Ab2, Cry1F, PAT, and CP4 EPSPS	2010
PowerCore™ Ultra	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6 × SYN-IR162-4	HT and IR	Cry1A105, Cry2Ab2, Cry1F, PAT, CP4 EPSPS, and Vip3Aa20	2017
VT PRO3®	MON-89Ø34-3 × MON-88Ø17-3	HT and IR	Cry1A.105, Cry2Ab2, Cry3Bb1, and CP4 EPSPS	2011
VT PRO2®	MON-8746Ø-4 × MON-ØØ6Ø3-6	HT and IR	Cry1A.105, Cry2Ab2, and CP4 EPSPS	2010
Leptra®	DAS-Ø15Ø7-1 × MON-ØØ81Ø-6 × SYN-IR162-4 × MON-ØØ6Ø3-6	HT and IR	Cry1F, Cry1Ab, PAT, VIP3Aa20, and CP4 EPSPS	2015
VIP3®	SYN-IR162-4 × SYN-BTØ11-1 × MON-ØØØ21-9	HT and IR	VIP3Aa20, Cry1Ab, PAT, and mEPSPS	2010

Source: CTNBio - http://ctnbio.mctic.gov.br/
HT: Herbicide Tolerance; IR: Insect Resistance

is essential to their survival (Duke and Powles, 2008). Corn events tolerant to glyphosate, MON-ØØØ21-9 (Monsanto, 1997) and MON-ØØ6Ø3-6 (Monsanto, 2000), relies on the overexpression of mEPSPS [modified 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme] and CP4 EPSPS proteins, respectively. Both proteins have low affinity for biding glyphosate herbicide and, consequently, confer tolerance to over-the-top applications since they are functionally equivalent to native EPSPS. *CP4 EPSPS* genes was isolated from *Agrobacterium tumefaciens* and *mEPSPS* is a modified gene from corn, encoding an EPSPS protein with 99.3% of amino acid similarity with the native EPSPS from corn.

Auxin herbicides molecules, like 2,4-D (2,4-dichlorophenoxyacetic acid) and dicamba (2-methoxy-3,6-dichlorobenzoic acid), are similar to the natural auxin indole-3-acetic acid (IAA), triggering a similar but excessive cellular response, leading to uncontrolled plant growth and main commercial applications to control broadleaf weeds. The key receptor for auxins is located at the plasma membrane and named

Auxin Binding Protein1 (ABP1). Herbicide binding induces extracellular pH decline by pumping protons between symplast and apoplast (Tromas et al., 2010), leading to cumulative K^+ and water influx, increasing cellular turgidity (Maeshima, 2001). These changes lead to cell cytoskeleton alteration, reducing antioxidative defense, and then triggering ROS (Reactive Oxigen Species) overproduction, which causes cell wall reorganization. Due to the loss of cell wall structure, ROS are able to penetrate into the plasma membrane where they can interact with phospholipids promoting unsaturation of plasma membrane lipids, escape of the cytosol content, leading to cellular death (Jacob et al., 2015). Corn events DAS-4Ø278-9 and MON-87419-8 possess tolerance to 2,4-D and dicamba herbicides, respectively. DAS-4Ø278-9 corn expresses an aryloxyalkanoate dioxygenase (AAD-1) enzyme which degrades 2,4-D into herbicidally-inactive 2,4-dichlorophenol (DCP). Also, plants expressing AAD-1 have been demonstrated to convert certain AOPP herbicides (quizalofop, cyhalofop, haloxyfop) into their corresponding inactive phenols (Wright et al., 2010). Dicamba tolerance in MON-87419-8 corn was achieved by expression of dicamba O-demethylase (DMO) enzyme. DMO is a Rieske oxygenase that catalyzes an exocyclic monooxygenation reaction that leads to degradation of dicamba to non-herbicidal 2-hydroxy-3,6-dichlorobenzoic acid (D'Ordine et al., 2009).

Ammonium glufosinate [D,L-phosphinothricin or 2-amino-4-(hydroxymethylphosphinyl) butanoic acid] is a broad-spectrum herbicide that inhibits glutamine synthetase enzyme, preventing ammonia metabolism and resulting in the inhibition of photosynthesis in plants and subsequent leaf chlorosis (Takano and Dayan, 2020). Phosphinothricin acetyltransferase (PAT) is an enzyme that catalyzes the conversion of phosphinothricin into N-acetylphosphinothricin, a compound with no herbicide activity. Therefore, since PAT detoxifies ammonium glufosinate, its heterologous expression confers to corn cultivars tolerance to this herbicide (Botterman et al., 1991). Several corn technologies containing ammonium glufosinate tolerance are available for growers in Brazil, including Herculex® (DAS-Ø15Ø7-1) and LibertLink® (ACS-ZMØØ3-2) technologies.

Insect Resistance Traits

Insect control in corn fields is probably one of the most challenging topics in pest control. Insecticide proteins were first isolated from *Bacillus thuringiensis* (*Bt*), a ubiquitous gram-positive soil bacterium, from several sources including soil, leaves, sewers, and dead insects. *Bt* cells are able to produce crystals during sporulation phase, with a well-documented specific activity against several insect orders (Fernández-Chapa et al., 2019). Biotechnology industries were able to isolate genetic coding sequences for specific crystal proteins and heterologous express in transgenic corn events, giving origin to insect resistance traits. The first

GM plant expressing a *Bt* protein reached the market in the 1990s, however *Bt* based pesticides have commercially used since 1938 (Osman et al, 2015).

Despite insect resistant *Bt* crops having been proven to be a highly viable alternative to agrochemicals to control target insect pests, just a few insecticide proteins are available in the Brazilian market currently (Table 1). High regulatory costs for bringing a new trait to market (McDougall, 2011), aligned with difficulties in finding toxins with new mode-of-action (Heckel, 2020) results in a market with few choices for farmers, where most high performing technologies are based on combinations of existing technologies by conventional breeding. The first insect resistant corn hybrids were registered for commercial use in Brazil in 2008 and contained the gene *Cry1Ab* (YieldGard® and Agrisure®). In 2009, hybrids containing the gene *Cry1F* (Herculex®) was registered and in 2010 the first hybrids Viptera™ (*Vip3Aa20* gene) and YieldGard VT PRO® (*Cry1A.105* and *Cry2Ab2* genes) were registered (https://sistemas.agricultura.gov.br/snpc/cultivarweb/cultivares_registradas.php). Since 2010 there have been no more records of corn hybrids containing new proteins. Only hybrids containing combinations of existing proteins. Currently, top selling transgenic corn hybrids in Brazilian market contain a combination of insecticidal proteins to amplify product efficacy and avoid emergence of resistant insect populations (Table 2).

Recently available corn technologies to control target pests include expression of DvSnf7, a double strand RNA (dsRNA) that targets Snf7 gene leading to Western Corn Rootworm mortality (present in MON-87411-9 corn) and expression of Cry1Da_7 and chimeric Cry1B.868 proteins (Wang et al., 2019), as found in MON 95379 corn (Table1).

Given the combination of both insect resistance and herbicide tolerance traits, outstanding benefits from biotech crop adoption were obtained in Brazil. It is estimated, from 2003 to 2016, the incomes from biotech adoption in Brazilian fields exceed US$ 19 billion (Brookes and Barfoot, 2016). Profits obtained per hectare from utilizing biotech corn hybrids was up 64% in the summer season and 152% in the winter season compared with conventional hybrid. The volume of pesticides applied per hectare was reduced by 18% and 16% for summer and winter season corn harvests, respectively, which corresponds to 91 million tons. This decrease in agrochemical applications indirectly resulted in significantly reducing the impact for workers, animals, and environment, reducing fuel consumption by 110 million liters. In 20 years of adoption of biotech corn worldwide, production increased by 75% while planted area only increased by 18%, resulting in an 80% revenue increase (Council for Information on Biotechnology, 2018).

The benefits of GM corn for Brazilian agriculture are evident and endorsed by solid scientific literature and robust data. Those benefits extend from gains in yield, lowering costs, and reducing human/environmental risks extending

to outcomes directly related to improving farmers management conditions in Brazilian producing fields. Biotechnology will certainly continue to revolutionize agriculture and improve people's lives.

Insect Resistance Management

The large benefits from *Bt* crops are constantly threatened by the emergence and selection of insect populations resistant to *Bt* proteins. In Brazil, Cry1Ab and Cry1F toxins were present in first insect resistant corn cultivars, mainly represented by YieldGard® and Herculex® corn technologies. Despite presenting significant efficacy against fall armyworm (FAW – *Spodoptera frugiperda*) (Omoto et al., 2016), Cry1Ab resistant population started to emerge from Brazilian fields 3-4 years after starting commercialization (Fatoretto et al., 2017). The same timeline was observed for emergence of Cry1F resistant populations in Brazil (Farias et al., 2014). In both cases, the fast emergence of resistance can be attributed to MON-ØØ81Ø-6 (Lynch et al., 2003) and DAS-Ø15Ø7-1 (Monnerat et al., 2015) corn events are not high-dose products against FAW (Sousa et al., 2016).

Diverse proteins have been proposed as receptors for *Bt* toxins, including ATP-biding cassette (ABC) transporters, cadherins (CAD), alkaline phosphatases (ALP), and aminopeptidases (APN) (Bravo et al., 2007). ATP-binding cassette (ABC) type 2 transporters seem to be the receptors for several Cry1 proteins, including Cry1A (Gahan et al., 2010) and Cry1F (Wang et al., 2020). ABC transporters are membrane-associate ATP-dependent proteins involved in transporting several substrates including lipids, peptides, amino acids, sugars, and xenobiotics (Wu et al., 2019). Mutational disruption of ABCC2 in FAW confers resistance to Cry1F and Cry1A.105 (Flagel et al., 2018) but no resistance was observed for ABCC3 mutation (Jin et al., 2021). The sequencing of ABCC2 gene from two Cry1F field resistant populations in Brazil identified a GY deletion (positions 788-789) and a P799K/R substitution at the extracellular loop 4 (Boaventura et al., 2020). In addition to loop4, ABCC2 loop 2 is also seems responsible for Cry1F insecticidal activity (Liu et al., 2021), probably serving as the toxin binding site.

Besides the desirable high-dose levels of *Bt* proteins present on corn cultivars, other insect resistance management (IRM) practices such as trait pyramiding and refugee adoption are needed to prolong the efficacy of insect control technologies. Trait pyramiding consists of stacking multiple *Bt* genes with different mode of action (MAO) or multiple technologies like *Bt* proteins and RNAi (Bolognesi et al., 2012). The purpose of pyramiding different technologies is to obtain negative cross-resistance, which can be observed in insect populations resistant to a specific *Bt* protein and susceptible to other *Bt* proteins, both present in the same cultivar (Pittendrigh et al., 2013). The opposite would be the positive cross-resistance, which is found on insect populations that are resistant to one

Bt protein that also exhibit resistance to other *Bt* proteins, usually by toxins that share similarities on the protein structure and possibly the same receptor on target pests (Carrière et al., 2015). Most current corn cultivars available for Brazilian growers count on three different families of *Bt* proteins, Cry1, Cry2, and Vip3Aa. For corn, Cry1 clade includes Cry1Ab, Cry1A.105, and Cry1F, while Cry2 and Vip3A comprise Cry2Ab2 and Vip3Aa20, respectively.

Stacked technologies that received regulatory approval from CTNBio in Brazil includes PowerCore™ Ultra, VT PRO3®, and Leptra®, and all rely on trait stacking for efficient insect control. As demonstrated early, field evolved Cry1F FAW tolerant populations, found in Brazilian fields, are still susceptible to Cry2A toxin, since they do not compete Cry1F to bind membrane vesicles (Monnerat et al., 2015). Insect bioassays indicate that stacking Cry1, Cry2, and Vip3Aa is an interesting strategy for insect resistance management for FAW (Gilreath et al., 2021). However, despite this combination of technologies being efficient against main lepidopteran pests, this practice alone does not guarantee the longevity of these technologies and other IRM practices are highly recommended. The adoption of corn germplasm with native genetic resistance and planting refuge are good practices to engage in order to extend the longevity of *Bt* technology efficacy.

Native insect resistance in plants consists on the employment of constitutive or inducible defenses mechanisms that are triggered by herbivore attack. Biological responses varies from the presence of indirect defenses leading to the lack of insect feeding preference, in general by releasing volatile compounds, to direct defenses mechanisms that requires the expression of defensive proteins, production of secondary metabolites, and any other compounds toxic to insects (Howe and Jander, 2008). Mp708 is a corn inbred line resistant to FAW larvae feeding that was developed using conventional breeding methods. This line presents a constitutive expression of jasmonic acid (JA) while Tx601, a genotype susceptible to FAW, only recruits JA pathway after insect feeding. Mp708 constitutively produces (E)-β-caryophyllene, a terpenoid frequently linked with resistance and acts by repelling FAW from feeding (Smith et al., 2012). The induction of defensive genes also plays important roles for controlling insect damage. Ribosome-inactivating protein 2 (RIP2) expression is largely induced in corn by insect larvae feeding but not by mechanical wounding. RIP2 expression is also influenced by several phytohormones, including methyl jasmonate, ethylene, and abscisic acid and its presence in corn leaves can retard caterpillar growth (Chuang et al., 2014). These natural mechanisms found on corn reiterates the importance of identification and combination of different native resistance mechanisms into corn germplasm that will be the basis for the introgression of *Bt* traits. This strategy seems to be also a viable option to increase *Bt* product efficacy and contribute to avoid resistance

emergence.

Refuge strategy consists of planting a structured area of non-*Bt* crops, and has been shown to be valuable for both singles and pyramided *Bt* products (Carrière et al., 2020). These areas are hosts to insects that are susceptible to *Bt* toxins, and therefore contribute to maintaining susceptible allele from *Bt* susceptible individuals, reducing the formation and selection of homozygous-resistant individuals. Usually, this strategy works best if the resistance mechanism is recessive (Carrière et al., 2016). In Brazil, CTNBio does not regulate IRM practices and adoption is not mandatory. Unfortunately, despite several attempts at cooperation between academy and industry, creation of initiative groups and guidelines, the adoption of refuge areas by growers remains low (Fatoretto et al., 2017). The conscientization of farmers to emergence of resistance risks, creation of robust IRM requirements, establishment of resistance monitoring programs, and clear corrective actions are steps required to maintain the outstanding benefits of insect resistant crops.

Biotechnology, Regulatory and Breeding GM Corn Hybrids

Three distinct and equally important phases comprise the GM cultivar process: a) discovery and obtaining of the elite trait by the biotechnology team; b) biosafety evaluation of the new trait for its deregulation by the regulatory team; and c) obtaining GM cultivars containing the new trait by the breeding team (Figure 2). The first two steps are performed only once for any new trait, while the breeding of GM plants with this new trait is continuous (Schuster, 2017).

The Biotechnology and Regulatory stages need to follow biosafety laws and Biosafety Agency rules. In Brazil, as mentioned before, the Biosafety Agency is CTNBio, and only Legal Entities can work with GM organisms before deregulation. All institutions that wish to work with regulated GM organisms need to have a Certified of Quality in Biosafety (CQB) (Law 11105, 2005).

In the discovery phase, numerous candidate genes for the target characteristic are transferred to model plants (tobacco, *Arabidopsis*) or target species. The genes with potential to be used in commercial crops are introduced, by plant transformation, to target species (corn, for example) to perform the proof of concept (Phase I). In the proof of concept, the expression of the desired trait such as herbicide tolerance, insect resistance or otherwise, is evaluated in the target plant. The main objective is to evaluate whether the expression of the gene in the target plant produces enough protein, and if the desired characteristic manifests itself with high efficiency. In this stage, many transformation events with the target trait are obtained, to select an elite trait. The elite trait should contain a single copy of the insert, express itself efficiently and not cause any changes in the transformed plant, in addition to the change desired by the transformation. Most part of candidate genes are discarded in this stage

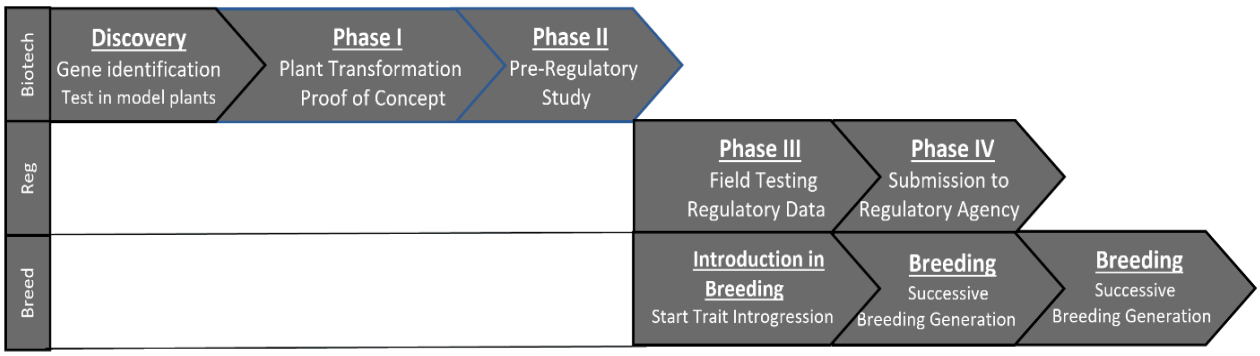

Figure 2. Steps to obtain GM cultivars, from the identification of the gene of interest to the breeding of plants with this new trait. This model is similar for all GM crops (Schuster, 2017).

(Schuster, 2017)

After selecting the elite event with the desired expression of GM trait, the GM event need to be evaluated regarded its biosafety. In the regulatory stage it is necessary to obtain experimental data to demonstrates the food, feed, and environmental safety of the new GM trait. This data needs to be submitted to the regulatory Agency (CTNBio in Brazil). The regulatory Agency evaluates the data and decides whether to approve the new GM plant. In Brazil, CTNBio evaluates aspects related to the biosafety of the new trait. In case of approval by CTNBio, it is still necessary to obtain the approval of CNBS (National Biosafety Council), which evaluates the new trait from the point of view of market opportunity and risk (Law 11105, 2005).

If some breeding activities starts before the commercial approval of the new GM event, these activities need also to follow the biosafety rules. In case of breeding activities in greenhouse, it needs to be approved by the institution internal biosafety committee (CIBio), and in case of field breeding activities, it needs to be approved by CTNBio (http://ctnbio.mctic.gov.br/resolucoes-normativas). As the submission and approval of field activities with regulated GMO traits takes time, these activities need to be planned some months in advance to avoid the risk of losing the preferred planting season. Regulated tests are inspected by the Ministry of Agriculture, IBAMA (Brazilian Institute for the Environment and Renewable Resources) and could be inspected by ANVISA (National Health Surveillance Agency), which assesses whether all biosafety measures contained in the LPMA request for conducting the tests have been met.

Once GM events are approved for commercial use, CTNBio approval is no longer required for field improvement activities. That is because the commercial approval of GM events attests their biosafety. But it doesn´t means the breeding of GM corn is the same as breeding conventional corn. Schuster (2017) described

the impact of breeding GM plants in soybean breeding. In autogamous species, the introduction of GM traits in breeding varieties results in an initial reduction in genetic variability and increase in breeding population size. In hybrid breeding, like in corn, the impact is different, and depends on breeding strategies.

The most common strategy for GM plant breeding in breeding varieties is to use forward breeding, i.e., to introduce the GM events in the breeding populations by crossing GM plants with conventional or GM plants. In hybrid breeding, the most common strategy for GM breeding is to develop the basic germplasm in the conventional way and convert the parental lines of commercial hybrids with the GM trait, using backcross approach. As the GM traits is normally dominant, just one parent of each hybrid needs to be converted.

Trait introgression based on Marker Assisted Backcross (MABC) is the most common approach to introduce GM traits in corn hybrids (Figure 3). MABC can introduce the GM trait, as well as native traits, in corn and recover the most part of recurrent genome (around 98%), commonly in two or three generations of backcross, depending on the number of traits (authors data, not shown). Considering all generations of backcrosses, the completion and test of converted inbreed lines as well as seed increase, the time to introduce a GM trait and produce the GM hybrids can vary from four to five years. To avoid the delaying in launching new hybrids with GM traits, backcross for trait introgression is normally started when a candidate elite inbreed line is identified in breeding program. Thus, when the hybrid is commercially advanced, at least one parent is already converted to the GM event.

Currently, the commercial GM corn in Brazil have from two to four GM insertions, mainly to confer insect resistance or herbicide tolerance (Table 2). The strategy for introducing multiple GM traits into a corn hybrid defines the design of the backcross program and the strategy also depends on the number of traits to be introduced. In a backcross program, in each generation of backcross it is expected half the plants to be heterozygous (or hemizygous, when the trait is GM) and half plants to be homozygous for the recurrent parent genotype, in the loci containing the target trait. Considering multiple GM traits, the expected number of plants hemizygous for all traits is $(1/2)^n$, where n is the number of GM traits. It means, for each new trait included in backcross program, the population size doubles.

Frisch et al (1999), in simulation studies, concluded that for better efficacy in selecting recurrent genome recovery in MABC, 100 plants are enough in each backcross generation. In real MABC program, for convenience, considering the PCR lab plates containing multiples of 96 samples, and considering the needs of controls in lab analysis, a multiple (or sub-multiple) of 90 plants can be considered. As the number of traits increase, the population size also increases, and with four or five traits it has a dramatic increase in BC population size

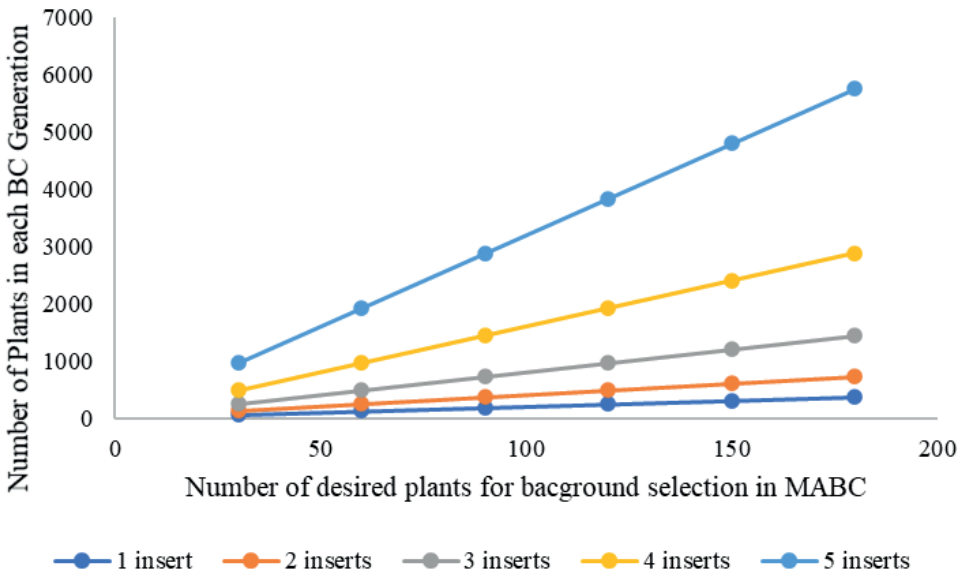

Figure 3. Population size in Marker Assisted Backcross program as function of the number of inserts to be introduced by backcrossing, and the desired number of plants with traits to be used for background selection.

(Figure 2).

With multiple traits, the Trait Introgression by MABC can be made in both parents of the hybrid, introducing part of the traits in the male parent and part of the traits in the female parent. Introducing part of the traits in one parent an part in other parent, the population size can be reduced in 50% in the case of four inserts, and the reduction in population size can reach 67.5% in the case of five insertions (Figure 4).

On the other hand, as introducing traits in two parents means having two parents in the backcross program, and the activities related to completion and test of converted inbreed lines and seed increase of new parent version is twice as the activities when just one parent is converted.

The introduction of new GM traits in corn breeding also potentially is associated with a reduction in the speed of genetic gain. When a new GM trait is introduced in a corn breeding program, it is expected the current best testers will be converted first. It means the same testers will be used for some more years. New good candidates for testers, as they have not yet been well proven, will be converted after the well-known testers. As a result, the change in the testers, or the use of new testers, can be delayed, resulting in delaying the genetic gain associated with the new tester. The alternative to avoid this delay in genetic gain is to convert as many parents as possible, especially testers, at the same time. But it is also associated with the increase in breeding costs.

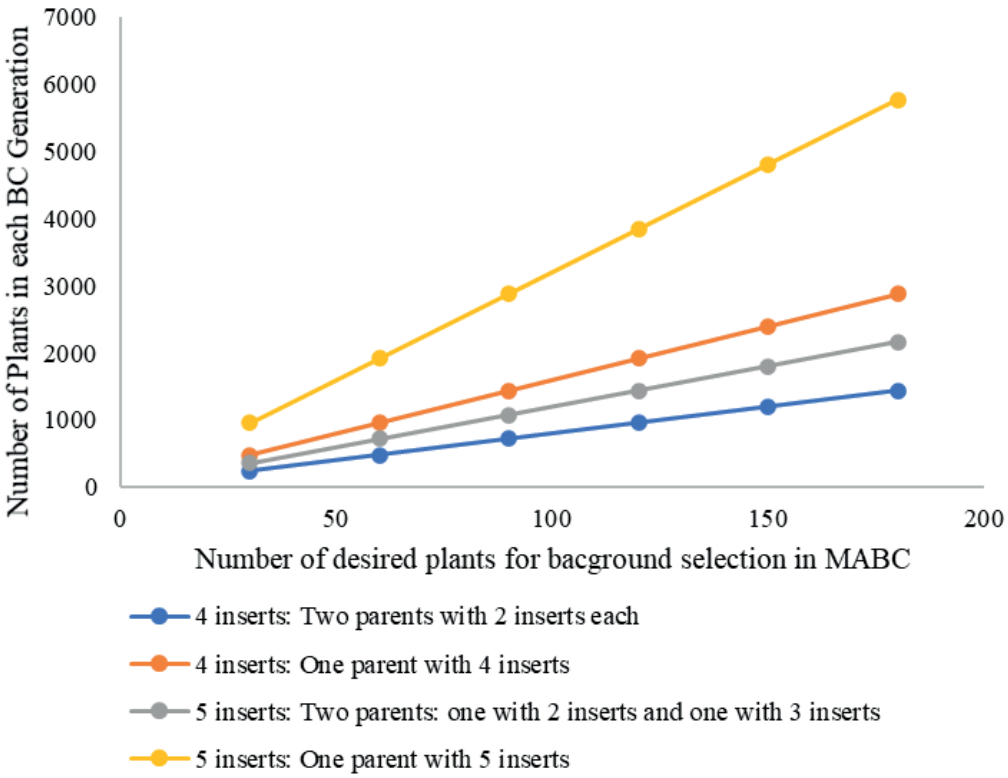

Figure 4. Population size in MABC approach considering all traits in one parent or splitting the traits in both parents of a GM hybrid.

Future Prospect

Over the past 25 years GM corn hybrids have become the dominant technology in the main producing countries. This technology has brought numerous benefits, but so far, the main applications are restricted to herbicide tolerance and insect resistance. Insect-resistance strains to GM traits have also emerged for some genes, and even with insect-resistance traits stacked in GM hybrids, the efficacy of these traits is not expected to be long. It is because the current stacked traits have a small number of modes of action. In recent years, some insect-resistance traits with new mode of actions were developed, as Cy1B, Cry1D and dsRNA events (Table 1). But new traits for insect resistance will be every time more difficult to be found by the same way the first generations of insect-resistance genes were discovered and used in GM plants. It is also the same for other traits in corn and other species of plants. New biological and genetic engineering approach will be added to the current approach to create new valued traits for crop plants. Some of them with the potential to replace GM technology.

With the availability of complete genomic

sequence databases for various crop species, in line with a regulatory favorable scenario perspective, genome editing technologies are emerging as powerful tools to precisely edit genomic sequences and produce valuable improved agronomic products. So far, the most used technology named CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is based on the premise of using a nuclease driven by RNA guides to generate a precise double-strand break, followed by non-homologous end joining (NHEJ) or homology direct repair (HDR). However, several other applications of this technology can be found in agriculture (Zhu et al., 2020).

In Brazil, products obtained using CRISPR and any other so called new precision breeding technologies, are evaluated in a case-by-case consultation scenario, where CTNBio deliberates the product status (GM or non-GM) according to Normative Resolution No. 16, from January 15th, 2018. To date, the only corn product consult submitted for CTNBio appraisal was waxy corn from DuPont Pioneer (Johnston, Iowa, USA). This waxy corn starch is composed mainly of amylopectin, which was obtained by inactivating the endogenous Wx1 waxy gene with CRISPR/CAS9. Wx1 gene encodes a granule-bound start synthase that is involved in the production of amylose (Waltz, 2018). After evaluation, CTNBio deliberated to consider waxy edited corn a non-GM product (CTNBio internal process no. 01250.033737/2018-67). Other important agriculture countries, like USA and Argentina use the same approach of case-by-case evaluation. Although, in EU (Europe Union), traits obtained by genome editing are considered GMO.

In a similar context, synthetic biology (SynBio) is emerging as a promising set of technologies with potential to revolutionize agriculture. SynBio is the use of directed evolution for designing, evolving, and selecting new biological components, such as molecules, enzymes, genetic material, microbes, and cells. Despite the huge potential in agriculture, there are few examples of utilization for plant improvement, once bacterial, yeast, and mammalian systems present many more advances and industry applications (Roell and Zurbriggen, 2020). Phage-assisted continuous evolution (PACE) has been employed for overcoming insect resistance. Evolved variants of Cry1Ac has been selected to bind *Trichoplusia ni* cadherin-like receptor and make this non-susceptible specie susceptible to evolved Cry1Ac (Bradan, 2016). This type of application opens several possibilities for large-scale designing synthetic toxins for target insect pests without relying on finding and isolating natural sources.

Nitrogen is a limiting nutrient for plant growth and its availability has a direct effect on crop yield. SynBio techniques has been employed to replace *Escherichia coli* genetic chassis with ferredoxin-NADPH oxidoreductases (FNRs) and ferredoxins molecules derived from plant organelles, reducing the required components for fixing nitrogen. FNR-ferredoxin module

originated from organelles seems to be functional in both endogenous and modified nitrogenase enzymes, raising future opportunities for diazotrophs engineering in corn and obtention of a N-fixing plant (Yang et al., 2017). Crops requiring less use of agrochemicals are in high demand, especially for industrial fertilizers, which production have been associated to environmental pollution and expressive manufacture costs.

Ambitious projects are currently aiming the utilization of SynBio to rebuild entire yeast genome, synthetizing and mounting each chromosome (Richardson et al., 2017). These forms of basic research help to elucidate the most fundamental principles of life chemistry, making possible the proposal of until then distant applications like producing a synthetic chloroplast genome (Piatek et al., 2018). Chloroplasts are the core of photosynthesis and also target of multiples virus in plants (Zhao et al., 2016). Corn chloroplast genome has only 140 kb in length and contains 118 genes, which makes straightforward a complete redesignation, when compared to nuclear genome (Chen et al., 2020). New research groups are starting to emerge in both industry and academia environments, applying SynBio for creating innovative proposals and solutions for problems facing by farmers from planting to harvest. However, a global regulatory alignment and synchrony must be pursued to these new set of technologies thrive, and final products reach every potential costumer without extra regulatory costs and approvals delays.

Final Considerations

GM corn already completed 26 years in USA, and 14 years in Brazil. The adoption of GM corn in Brazil was extremely fast, requiring only a few years to reach 80% of the planted area. The benefits for the farmers and for the environment with the use of GM corn was enormous. But after 25 year of GM corn, the main traits being used are herbicide tolerance and insect resistance. The last commercial insect-resistance toxin was launched in Brazil in 2010. The risk to have insect resistant strains for all commercial traits in Brazil is high, and the industry are working to develop new solutions.

The development of GM traits for other important applications, like yield improve, abiotic stress tolerance, better food and feed quality, broad spectrum of disease resistance, and others, are still being expected by the farmers. As those kinds of traits are being difficult to be obtained by current approach of GM technology, new emerging technologies like genome editing, synthetic biology, and other new technologies can be a way to reach this objective.

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