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U.S. EPA Regulation of Plant-Incorporated Protectants: Assessment of Impacts of Gene Flow from Pest-Resistant Plants

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ABSTRACT: The U.S. Environmental Protection Agency licenses pesticide-expressing plants under the authority of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Transgenes and their pesticidal products represent pesticides under FIFRA and are referred to as plant-incorporated protectants (PIPs). When sexually compatible wild relatives (SCWR) are sympatric with PIP crops, there is a need to assess the potential for adverse effects to man and the environment resulting from transgene introgression in accord with FIFRA requirements. Genetic compatibility, introgression, weediness of SCWR × PIP hybrids, seed dispersal, and dormancy, among other parameters, as well as effects on other species (herbivores and beneficial insects), all need to be considered as part of the risk assessment for experimental use under Section 5 or registration under Section 3 of FIFRA. EPA is currently developing data requirements and guidance toward addressing potential gene flow impacts from PIPs.

KEYWORDS: EPA (U.S. Environmental Protection Agency), FFDCA, FIFRA, plant-incorporated protectant, gene flow

INTRODUCTION

All plants produce forms of protecting compounds to preclude or reduce the incidence of microbial colonization, infection, or herbivory. These naturally occurring pesticidal substances range from antimicrobial compounds (e.g., saponins, glycoalkaloids, flavonoids) to signaling molecules (e.g., pattern recognition receptors) to plant growth regulators (e.g., jasmonic acid, brassinosteroids) to physical barriers (e.g., cutin, suberin). Although these compounds have been one focus of classical plant breeding for over a century, their precise roles and applications continue to evolve as does our understanding of their actions. When pesticidal substances meeting the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) definition of a pesticide are applied to plant defense or plant growth regulation through genetic engineering, they are typically considered to be plant-incorporated protectants (PIPs).

The U.S. Environmental Protection Agency (EPA) regulates the sale, distribution, and use of all pesticides in the United States. Pesticides include synthetic conventional chemicals, naturally occurring biochemicals, microbial agents, and pesticidal substances expressed in plants (i.e., PIPs). PIPs include the pesticidal substance(s) expressed, as well as the genetic material necessary for their expression. To date, the majority of registered PIPs have consisted of one to a few transgenes expressing insecticidal proteins, such as Cry and Vip proteins from *Bacillus thuringiensis* (*B.t.*).[1]

Higher plants, including crops and wild relatives, routinely exchange genes through hybridization, which results in introgression of genetic material into the genome of the recipient (female, seed) plant. A variety of mechanisms, both physical and biological, serve to effect this pollen-mediated genetic exchange or to prevent it. Proximity of compatible species, wind- or animal-mediated pollination mechanisms capable of sufficient transport distances, and a phenological overlap in flowering or nick are all important parameters in hybridization potential between two plants. Genetic barriers, including self-incompatibility (SI) alleles,[2] manage the compatibility reactions of pollen grains and styles of many higher plant species. When compatible interactions between pollen and stigmatic surfaces occur, hybridization may lead to gene introgression and expression in the recipient plant population. The introgressed transgene(s) may encode pesticidal traits (e.g., disease or pest resistance, plant growth regulation). If the action of the PIP transgene results in what is determined to be an unreasonable adverse effect upon man and the environment, then action under the oversight of FIFRA as pesticides (FIFRA 2(u)) would be warranted. The EPA previously exempted naturally occurring plant pesticidal substances, as present in all plants, from FIFRA oversight.[3]

Whereas gene flow contributes naturally to the evolution of species, the introgression of genes from transgenic crops into sexually compatible wild relatives (SCWR) may cause adverse effects on biodiversity.[4] The movement of transgenes from crop species to SCWR may impart a biological impact upon the recipient plant directly[5] and also on the ecosystem as a whole through community-related effects.[6,7] Such gene flow could also have socioeconomic effects if, for example, the spread of the transgene compromised the market value of some other crops. As such, the potential for adverse environmental impacts falls under the oversight of FIFRA and must be part of the risk assessment conducted during issuance of experimental use permits and registration for commercial use of regulated PIPs.

EPA ASSESSMENT OF GENE FLOW

Risk assessors need to consider the SCWR present in the areas where PIP plants are intended for cultivation. It is envisioned that

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over time, the spectrum of traits deployed and the variety of species expressing pesticidal traits will grow, requiring wide-ranging consideration of plant and trait characteristics in different environments. To date, the predominance of the registered PIPs are expressed in few species. PIP product development is limited to maize, cotton, potato, and a single PIP registration in European plum (recently completed).

Maize (*Zea mays* L.). Critical to any understanding of gene flow events and impacts is the geographic distribution of SCWR in the regions wherein the PIP plant will be cultivated. For maize, the absence of wild forms of related congeners in the United States precluded the need for further risk assessment into the consequences of gene flow between registered PIPs and wild relatives.[8] With the exception of some special plantings of teosintes (i.e., various *Zea* species) for research or demonstration purposes, the exposure of any SCWR to pollen derived from PIP-expressing cultivars of maize is extremely remote in the United States. These special plantings are highly managed cultivations with little probability of the establishment of self-sustaining populations.

The only other candidate SCWR known for the genus *Zea* are the *Tripsacum* species (*T. dactyloides* (L.) L., *T. floridanum* Porter ex Vasey, and *T. lanceolatum* Rupr. ex Fourn.), which grow in the United States as feral populations or managed plantings for forage purposes. *T. floridanum* is known from southern Florida, and *T. lanceolatum* is present in the Mule Mountains of Arizona and possibly southern New Mexico; neither of these species is widely planted or managed for food or feed purposes. *T. dactyloides*, Eastern gamagrass, is the only member of this genus that has some agronomic importance as a pasture/forage grass and is cultivated sympatrically with commercial maize.[9]

The only known case of a naturally occurring *Zea*−*Tripsacum* hybrid is a species native to Guatemala known as *Tripsacum andersonii* J.R. Gray, believed to have originated from the hybridization of *Zea luxurians* and *Tripsacum laxum*. It is 100% male and nearly 99% female sterile and is thought to have arisen from gene flow to teosinte, but the lineage is uncertain.[10] *Tripsacum andersonii* is also known as *Tripsacum fasciculatum* Trin. ex Aschers, Guatemalan gamagrass, and *Tripsacum laxum* Nash., by some authorities. This species and *Tripsacum latifolium* Hitchc., wideleaf gamagrass, are known from sites in Puerto Rico, but do not hybridize with maize or any of its closest relatives.[11−13]

On the basis of the absence of SCWR of maize in the United States and the lack of weedy characteristics within the species, no concerns regarding gene flow impacts are expected, according to currently available information.

Upland Cotton (*Gossypium hirsutum* L.). Upland cotton, *G. hirsutum* L., is known to have SCWR in the form of indigenous and feral populations of *G. hirsutum* and *Gossypium barbadense* L. in the Florida Keys, Puerto Rico, Hawaii, and the U.S. Virgin Islands. In many instances these indigenous populations exist as hybrid swarms of the two species and are difficult to distinguish phenotypically and genetically. Both species are allotetraploid ($4x = 52$) and capable of interbreeding with each other and feral escapes, which may represent intermediate forms.[14]

Feral populations of *G. hirsutum* exist in southern Florida, the U.S. Virgin Islands, and Puerto Rico, all areas within the Caribbean Basin and considered within the center of origin for the New World Gossypieae. Pima cotton, *G. barbadense*, is also found in the Caribbean, including the U.S. Virgin Islands and Puerto Rico. The semiwild cotton of the Virgin Islands may constitute an introgression of genetic components from *G. hirsutum* and *G. barbadense*.[15] Upland cotton, *G. hirsutum*, is genetically compatible with *G. barbadense* or Pima cotton and will produce viable, fertile progeny when crossed. Alleles specific to *G. barbadense* were found at a low frequency in feral *G. hirsutum* populations in the tropics and subtropics in areas where they are sympatric.[16]

The island of Puerto Rico is commonly used as a site for winter breeding nurseries of *G. hirsutum* due to the favorable climatic conditions during the winter months. Given the proximity of indigenous populations of *G. hirsutum*, *G. barbadense*, and hybrids of these two species, the EPA has instituted a 3-mile radius extending from the experimental test plots of *B.t.* cotton (e.g., breeding nurseries under Experimental Use Permit, Section 3 FIFRA) within which no feral or indigenous SCWR may exist. Additionally, 12 rows of phenologically similar non-PIP cotton must be planted surrounding the test plot to mitigate the potential for pollinator-mediated gene flow. This risk management decision reflects the recommendations of the EPA FIFRA Scientific Advisory Panel held in October 2000.[17] Briefly, the panel indicated that in situations wherein substantial necessary information regarding gene flow and its potential impacts is lacking, mitigation of gene flow potential is the appropriate action. Furthermore, the October 2000 Scientific Advisory Panel reported that assessment of the rate of gene flow was not directly relevant to the risk assessment because even very low levels of gene flow could lead to introgression, establishment of transgenes in SCWR populations and, potentially, subsequent impacts on the environment.

Arizona cotton, *Gossypium thurberi* Todaro (*Thurberia thespesiodes* Gray) occurs in the mountains of southern Arizona and northern Mexico at 750−1500 m (rarely at 2100 m) and is rather common on the rocky slopes and sides of canyons in late summer and autumn.[18,19] The diploid species *Gossypium thurberi* is not found in the areas where cotton is grown (i.e., desert valleys), and the progeny would be sterile due to their triploid state if gene flow and hybridization did occur with Upland or Pima cottons. Attempts to deliberately cross *G. hirsutum* with *G. thurberi* as the female parent have been unsuccessful. Additionally, the flowering periods of the commercial cotton and *G. thurberi* are primarily incongruous. Any gene exchange between plants of *G. hirsutum* and *G. thurberi*, if it did occur, would result in triploid ($3x = 39$ chromosomes), sterile plants because *G. hirsutum* is an allotetraploid ($4x = 52$ chromosomes) and *G. thurberi* is a diploid ($2x = 26$ chromosomes). Such sterile hybrids have been produced under controlled conditions, but they would not persist in the wild; in addition, fertile allohexaploids ($6x = 78$ chromosomes) have not been reported in the wild.

The second wild native species present in the United States in this genus, *Gossypium tomentosum* Nutt. ex Seem (Hawaiian cotton), occurs in Hawaii on the six islands of Kahoolawe, Lanai, Maui, Molokai, Nihau, and Oahu,[20] although it is rare on Molokai.[15,21] Upland, Hawaiian, and Pima cottons are all tetraploids ($4x = 52$) that can interbreed. Although originally thought to be pollinated by night-flying Lepidoptera, more recent observations indicate that the same pollinators that visit flowers of cultivated cotton are also capable of effecting pollination of *G. tomentosum* in its native habitat.[21] Introgression has been claimed for what one author considered hybrid swarms of *G. barbadense* × *G. tomentosum*, but conclusive proof of this is lacking. *G. tomentosum* is a tetraploid capable of forming fertile hybrids with *G. hirsutum* despite some fertility or compatibility factors.[22] Winter

nursery seed increases on any of these islands could result in further exposure of wild *G. tomentosum* to cultivated species, which will cross readily as all are tetraploids of the A−D genome type.

To date, it has been the policy of the EPA to prohibit the culture of *B.t.* cotton in Hawaii to mitigate potential gene flow events, as information regarding potential impacts of gene flow is insufficient and a comprehensive risk assessment has not been completed.

Potato (*Solanum tuberosum* ssp. *tuberosum*). Tuber-bearing *Solanum* species, including *S. tuberosum*, cannot hybridize naturally with the non-tuber-bearing *Solanum* species in the United States.[23] Three species of tuber-bearing (section Petota) wild species of *Solanum* occur in the United States: *Solanum fendleri*, *Solanum jamesii*, and *Solanum pinnatisectum*. Successful gene introgression into these tuber-bearing *Solanum* species is virtually excluded due to the constraints of geographical isolation and other biological barriers to natural hybridization.[24] These barriers include incompatible (unequal) endosperm balance numbers (EBN) that lead to endosperm failure and embryo abortion, multiple ploidy levels, and incompatibility mechanisms that do not express reciprocal genes to allow fertilization to proceed. No natural hybrids have been observed between these species and cultivated potatoes in the United States.

In the United States, *S. fendleri* (wild horsenettle) and *S. jamesii* (wild potato) are restricted to high-elevation habitats in the continental southwest, which are far removed from the centers of commercial potato production. Their distribution has been described in ref 19: (1) *S. fendleri* subsp. *fendleri* Asa Gray, Arizona, Colorado, New Mexico and Texas at 1600−2800 m in dry oak−pine forest, but not under dense shade. (2) *S. fendleri* subsp. *arizonicum* Hawkes, Arizona in pine forest clearings and roadsides from about 2000 to 2550 m. (3) *S. jamesii* Torr., Arizona, Colorado, New Mexico, Texas, and Utah.

If plants of *S. tuberosum* (commercial potato) and any of the three native tuber-bearing species were to grow contiguously, cytological differences in ploidy level and/or endosperm balance number between the wild and cultivated species would bar successful hybridization and gene introgression.[25] Controlled crosses between *S. fendleri* and *S. tuberosum*, for example, have been successful only with intermediate bridging crosses and have produced hybrids incapable of further sexual reproduction.[26] This does not present a risk of spread because intermediate bridging crosses do not occur in nature, due in large part to geographic and phenological separation.

With the significant geographic separation of commercial potatoes from wild species and the genetic factors affecting incompatibility, the probability of gene flow from transgenic potato to wild forms is extremely small in the United States. Although *B.t.* potato is currently registered as a PIP, there is no commercial acreage planted at this time.

European Plum (*Prunus domestica*). European plum, *P. domestica* cv. BlueByrd, was engineered by the USDA-ARS with the coat protein gene of plum pox virus to create the C5 Honeysweet Plum with resistance to the plum pox virus.[27,28] The non-native European plum, *P. domestica*, does not hybridize with native plum species in the Americas, such as *Prunus americana* Marsh., *Prunus alleghaniensis* Porter, or *Prunus angustifolia* Marsh. *P. domestica* is hexaploid ($2n = 48$, $x = 8$), and the American and naturalized or cultivated Asian species (e.g., *Prunus salicina*) are all diploids ($2n = 16$). These inherent ploidy differences preclude interspecific hybridization of *P. domestica* with native *Prunus* species. The absence of genetic compatibility with any extant plum populations in the United States alleviates any gene flow impact concerns.[27]

■ ASSESSING IMPACTS OF GENE FLOW EVENTS

With the progress made in genetic transformation methods and the advancement of biotechnological modifications of an expanding variety of crop plant species, the possibility of gene flow events involving transgenes becomes more likely. Methods of assessment for potential environmental impacts from gene flow continue to evolve; however, they are largely based upon models that need to be adapted to regional environmental conditions and the specific traits involved.[29−31] The scientific basis and information needed to perform an environmental risk assessment considering the movement of pesticidal traits into SCWR of crop species is currently being considered.[32]

The presence of traits that impart a selective advantage to plants represents a potential avenue for alterations in the population dynamics of a plant species recipient of these disease or pest resistance transgenes.[33] The rate of spread of the transgene(s) through plant populations will depend on the fitness of the hybrid, the fitness imparted by the transgene, and the geographic extent of the recipient species, as well as the frequency of use of the PIP crop expressing the transgene.[34] Alterations in allelic frequencies or actual swamping of a wild population with continued gene flow from domesticated crop species are of interest in environmental risk assessment, but do not a priori represent an adverse environmental impact. The standard within FIFRA is the advent of "an unreasonable adverse impact upon man and the environment" with the use of a pesticide product. Determination of what constitutes an unreasonable adverse environmental effect is based upon the parameters specific to the pesticidal product (e.g., trait, plant species, location, intended use) and is determined at the discretion of the Administrator of the EPA. FIFRA is a risk benefit statute and relies on a comparison of what environmental impacts and economic benefits may be realized or lost on the basis of the use of a proposed product registration in agriculture.

A variety of aspects of the crop plant species, PIP traits, SCWR, and effects on other associated species and biodiversity are to be considered when an environmental risk assessment is conducted. Although in theory any trait that provides resistance to a pest is potentially a selective advantage to a recipient species[35] and could alter population and community dynamics, in many instances such traits will not provide a significant advantage to the recipient population in the absence of pest or disease pressure necessary to effect that influence.[36,37] It is plausible that the initial introgression of a transgene may later be lost from the population due to a lack of selection[38] or fitness costs related to expression of the pest resistance trait[39] or may persist indefinitely as a "neutral" gene.[40]

The problem formulation stage of any gene flow assessment must consider the proximity of recipient populations of SCWR as well as phenological and genetic factors that may influence the potential for hybridization and introgression. An understanding of pollination mechanisms, pollinators, and pollen biology is important in establishing the capacity for hybridization of PIP-expressing species and their SCWR. Insect pollinators are known to exhibit various levels of specificity in flower selection and in their foraging range. Wind-pollinated plants may produce pollen that is relatively heavy and short-lived (e.g., maize) or which is smaller, lightweight, and capable of remaining viable for longer

distance transport and pollination (e.g., creeping bentgrass).[41] Knowledge of these factors is useful in cases when spatial separation of crops and SCWR is such that isolation distances can be used to mitigate gene flow. For some crops, such as sugar beet, the presence of isolated occurrences of SCWR in California and their absence elsewhere in the United States would allow strategic geographic deployment as an avoidance mechanism to preclude hybridization. Additionally, the biennial nature of this crop and its harvest during the first season contribute to a lowered probability of gene flow between crop and SCWR. In other instances, such as for the sunflower and the widespread occurrence of SCWR of this crop in the United States,[40,42] gene flow is inevitable unless a mechanism to restrict pollen fertility and outcrossing to SCWR is employed.

Demonstration of introgression and production of fertile, viable progeny, including stable inheritance and expression, mandate further investigation into the potential for environmental impacts resulting from movement of transgenes into SCWR. Following the determination of transgene introgression, the following parameters need consideration as part of the risk assessment:

Does the PIP transgenic trait engender a phenotypic trait that could confer a selective advantage (i.e., make the SCWR a better competitor within its natural habitat) in a wild population of SCWR or crop−wild hybrid?

Does the PIP transgenic trait enhance the weediness or invasiveness (i.e., increased vigor and fertility) of the plant that expresses the PIP?

Is the plant habit altered by the expression of the PIP transgene?

Are there effects on seed dormancy, viability, germination, fecundity, or dispersal ability as a result of PIP trait expression?

Are pest or disease organisms that are known to limit SCWR population growth (λ) present in the area of cultivation?

Do species listed as endangered or threatened and susceptible to action of the PIP trait exist in areas of cultivation?

Are the PIP plant species or SCWR listed on any state or federal noxious weed list?

Does introgression of the PIP trait into an SCWR population influence the phenological development of the SCWR (e.g., flowering period)?

Does the transgene product affect other plants, herbivores, or beneficial insects?

Test methods exist for the determination of gene introgression, assessment of plant habit and phenological alterations, and disease or pest resistance; however, assessment of larger scale population and community effects are more complicated and potentially problematic to quantify. As populations of SCWR and their associated community members vary regionally as well as temporally, understanding the dynamics of potential impacts on transgene recipients will require the establishment and use of baseline data and models to perform an adequate risk assessment. In some instances, semifield or mesocosm studies with small mixed-plant populations may provide valuable insight into the potential for interactions of SCWR and PIP-expressing species. Small-scale field experiments may be possible under an Experimental Use Permit from the EPA in instances wherein substantial isolation (e.g., spatial, temporal, genetic use restriction technology) precludes gene flow to SCWR populations. For longer lived perennials, such as trees, such experiments may be impractical and unworkable. The EPA will formulate experimental plans with the registrant of the product for assessing PIP-expressing plant species that have SCWR in the United States. Given the potential for variation between species involved, traits, and geographic deployment, it is clear that a case-by-case approach will be necessary.

During the problem formulation stage of the environmental risk assessment, the critical end points for interpreting any experimental or theoretical outcomes with regard to the FIFRA standard must be determined. Impacts per se are not a priori unreasonable adverse impacts that reach the FIFRA standard of action or trigger higher level tier testing and evaluation.[43] In addition to determining potential adverse effects associated with specific PIP traits and SCWR, it is important to determine how many generations of SCWR × crop hybrids need to be examined prior to registration or whether conditional registrations are needed to allow for additional experiments and observation as a form of environmental monitoring.

Gene flow between domesticated and wild plant forms has resulted in many intermediate hybrids and in some cases shifted the evolution of wild species and undoubtedly affected ecosystems to various degrees.[16,30,44,45] A key to estimating the potential consequences of gene flow events is the underlying background information of the species involved, including their basic biology and natural history. Crop plants that are highly domesticated are well researched and feature considerable published information from the public literature and databases regarding wild and feral relatives, as well as their distribution.[46,47] Traditional plant breeders also may have used some of the wild relatives in wide hybrid crosses and other breeding schemes, which further contribute to the knowledge base of plant characteristics. In contrast, species developed as PIPs for which much less is known about their relationship to any SCWRs (e.g., sexual compatibility) and for which even less is understood about the roles of these SCWR in a community context will be more difficult to quantify with respect to potential outcomes of gene flow events. These situations may require more extensive testing and experimentation prior to field release and registration.

To provide greater clarity to the registration process for PIPs, the EPA is presently delineating data requirements and associated guidelines for all aspects of product characterization, human health assessment, and environmental risk assessment through rulemaking. It is anticipated that the proposed rule (i.e., proposed data requirements for PIPs) will be available for public comment in 2011. This comment period will provide an avenue for input from all concerned parties and will aid the EPA in forming its final rule regarding gene flow data requirements and other aspects of PIP regulation.

Although this process is lengthy and requires both interagency input and public comment periods, the EPA continues in the interim to review PIPs on a case-by-case basis and maintain a science-based review process toward experimental use permit approvals (Section 5) and registrations (Section 3) under FIFRA.

This paper discusses the assessment of gene flow impacts in the United States and its possessions and territories under the authority of FIFRA. It must be recognized that although many of the concepts and metrics mentioned in this paper have applicability to the assessment of gene flow impacts regardless of where they occur, it is paramount that each country prepare its own risk assessment after careful consideration of the SCWR that may be present, as well as other relevant environmental parameters. Environmental variation, as well as biological and/or genetic differences of SCWR at regional and national levels, can make conclusions drawn under one set of circumstances inapplicable to other situations.

DISCLOSURE

The contents of this paper reflect the thoughts and opinions of the authors and do not represent an official policy statement from the U.S. Environmental Protection Agency or other federal government agencies. Any mention of a product does not constitute an endorsement by the U.S. federal government.

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