Appendix W. Plant Pest Risk Assessment for Glyphosate-Tolerant Alfalfa Events J101 and J163

Plant Pest Risk Assessment for Glyphosate-Tolerant Alfalfa Events J101 and J163

The APHIS Biotechnology Regulatory Service's (BRS) mission is to protect America's agriculture and environment using a dynamic and science-based regulatory framework that allows for the safe development and use of genetically engineered organisms. The regulations in 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason to Believe Are Plant Pests," regulate, among other things, the introduction (importation, interstate movement, or release into the environment) of organisms and products altered or produced through genetic engineering that are plant pests or that there is reason to believe are plant pests. Such genetically engineered organisms and products are considered "regulated articles." The regulations in § 340.6(a) provide that any person may submit a petition to APHIS seeking a determination that an article does not pose a plant pest risk and should therefore not be regulated under 7 CFR part 340. Paragraphs (b) and (c) of § 340.6 describe the form that a petition for a determination of nonregulated status must take and the information that must be included in the petition.

On April 16, 2004, APHIS received a petition from Monsanto Company and Forage Genetics International (Monsanto and FGI), requesting a determination of nonregulated status under 7 CFR part 340 for two alfalfa events designated as J101 and J163, which have been genetically engineered for tolerance to the herbicide glyphosate. This petition was assigned the APHIS number 04-110-01p, and will hereafter be referred to as "petition" in this Plant Pest Risk Assessment. APHIS evaluated the plant pest risks posed by the use of events J101 and J163 and included that analysis in an environmental assessment (EA) prepared to identify and evaluate any environmental impacts that could result from the deregulation of J101 and J163. In a notice published in the Federal Register on June 27, 2005 (70 *FR* 36917-36919, Docket No. 04-085-3), APHIS advised the public of its determination, effective June 14, 2005, that the Monsanto and FGI glyphosate-tolerant (GT) alfalfa events J101 and J163 did not pose a plant pest risk and were therefore no longer considered regulated articles under 7 CFR part 340. However this determination decision was later vacated in response to a lawsuit filed in the United States District Court for the Northern District of California that challenged APHIS' decision to grant nonregulated status to J101 and J163. On March 23, 2007, APHIS published a notice in the *Federal Register* (72 *FR* 13735-13736 APHIS Docket No. 04-085-1) announcing the Court's decision that the Monsanto and FGI GT alfalfa lines J101 and J163 were once again regulated articles under 7 CFR part 340. Because the determination decision was vacated, this plant pest risk assessment was conducted to determine whether GT alfalfa events J101 and J163 are unlikely to pose a greater plant pest risk than the unmodified organism from which they were derived.

Of the information requested by APHIS for submission of a petition for nonregulated status (§ 340.6(c)(4)), APHIS will use information submitted by the applicant related to plant pest risk characteristics, disease and pest susceptibilities, expression of the gene product, new enzymes, or changes to plant metabolism, weediness of the regulated article, any impacts on the weediness of any other plant with which it can interbreed, and the transfer of genetic information to organisms with which it cannot interbreed and subsequent plant pest impacts. Issues related to agricultural

or cultivation practices associated with herbicide use and its effects, and market and human health issues will be considered in the Environmental Impact Statement for alfalfa events J101 and J163 that is required as result of the Court order. These two alfalfa events are not genetically engineered to produce a toxin or pesticide, and are thus not targeted for use against pests in alfalfa agriculture. Therefore, APHIS did not examine the effects of the regulated articles on nontarget organisms. However, APHIS has examined the effects of alfalfa events J101 and J163 and potential use of glyphosate on animals, plants, and Threatened and Endangered Species (TES) and included the results of the analysis in the Environmental Impact Statement. APHIS has not identified any issues related to indirect plant pest effects on agricultural production caused by alfalfa events J101 and J163.

Potential impacts addressed in this risk assessment are those that pertain to the use of alfalfa events J101 and J163, and their progeny in the absence of confinement. The genetically engineered construct inserted into alfalfa to create events J101 and J163 was evaluated to determine if those sequences in J101 and J163 cause plant disease. Morphological characteristics of alfalfa lines J101 and J163 were analyzed to determine if these alfalfa lines would become weedy or invasive. The potential for gene flow to, and introgression of the transgene into, other alfalfa varieties or wild relatives of alfalfa was also evaluated to determine the potential for increased weedy or invasive characteristics in these plant species. APHIS also analyzed the propensity of alfalfa events J101 and J163 to become larger reservoirs of plant pests (insects or pathogens) compared to conventional alfalfa varieties, and the potential for horizontal gene transfer between alfalfa events J101 and J163 and bacteria and other organisms to lead to plant pest effects.

History of Development of Glyphosate-Tolerant Alfalfa Events J101 and J163

Alfalfa events J101 and J163 were engineered to be glyphosate-tolerant by inserting into the alfalfa genome a gene from the common soil bacterium *Agrobacterium* sp. CP4 that codes for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS), which unlike the native enzyme in alfalfa, is not inhibited by glyphosate. The gene was introduced into alfalfa using a disarmed *Agrobacterium*-mediated transformation system. The management of weeds in alfalfa fields can be an expensive, labor-intensive, and sometimes complicated operation. Often farmers use pre-emergent herbicides that will stop some weed seeds from germinating, and post-emergent herbicides that will control some weeds after germination. Typically, pre-emergent herbicide use is applied throughout the field because of the assumption that weeds will always be a problem in all parts of the field. Additionally, although these herbicides reduce weeds, most of these herbicides cause temporary damage to the desired alfalfa plants which results in reduced yields. With J101 and J163, and their progeny, farmers will have the option of applying the glyphosate herbicide after weeds have germinated only to areas of the field where there are weeds, and also to apply the herbicide without substantial damage to the alfalfa plants.

Glyphosate, a broad spectrum systemic herbicide, is one of the most environmentally friendly herbicides commercially available. The glyphosate herbicide (N-phosphonomethyl-glycine) is registered for non-selective weed control on both non-food use and food use plants.

Glyphosate works by interfering with normal plant metabolism by competing with the naturally present enzyme, 5-enolpyruvyl-3-phosphoshikimate acid synthase (EPSPS). EPSPS is involved in the biosynthesis of the aromatic amino acids, phenylalanine, tryptophan, and tyrosine (as well as some secondary metabolites) through the shikimate pathway. These aromatic amino acids are essential building blocks of proteins in all species. As a consequence of interfering with aromatic amino acid biosynthesis, plant cells cannot complete the synthesis of proteins and the plant dies (Kishore and Shah 1988). EPSPS is found naturally in all plants, fungi and some bacteria but is not present in animals (including humans). EPSPS from *Agrobacterium* sp. CP4 is less sensitive to interference by glyphosate (Barry et al. 1992), and therefore confers glyphosate tolerance to the genetically engineered alfalfa lines. For animals, aromatic amino acids must be obtained through the diet (Steinrucken and Amrhein 1980). Consequently, all animals are naturally exposed to sources of EPSPS through their normal diets.

Plant Pest Risk Assessment

Description of inserted genetic material and potential of the material to cause plant disease

Data supplied in the petition (Section V.A., pp. 38-68), and reviewed by APHIS, support the conclusion that lines J101 and J163 contain the following sequences: (1) a 35S promoter from a modified figwort mosaic virus (P-FMV), (2) coding sequence for a chloroplast transit peptide from *Arabidopsis thaliana*, (3) the 5-enolpyruvylshikamate-3-phosphate synthase gene (*epsps*) from *Agrobacterium* sp. strain CP4, and (4) DNA containing polyadenylation sequences from the 3' non-translated region of the *Pisum sativum* (pea) *rbc*S E9 gene. The non-coding 35S promoter is from the plant pathogen figwort mosaic virus. This sequence, however, cannot cause plant disease and serves a purely regulatory function for the *epsps* gene. The *epsps* gene is from the soil-inhabiting bacterial plant pathogen, *Agrobacterium* sp. strain CP4. It encodes the CP4-EPSPS enzyme which imparts tolerance to glyphosate. It does not cause disease and has a history of safe use in a number of previously deregulated genetically engineered plants (e.g., corn, cotton, soybean, rapeseed, and sugar beet varieties).

The plant material used for development of events J101 and J163 was Forage Genetics International's (FGI) proprietary alfalfa clone R2336 from a high yielding, fall dormant breeding population. The initial plants, selected for tolerance to glyphosate, were designated J101 and J163 (referred to in the petition as transformation events), and various populations were developed from these events to provide the data presented in the petition. Lines J101 and J163 were developed using a disarmed (i.e. pathogenicity genes removed) *Agrobacterium*-mediated transformation system on sterile alfalfa seedling cotyledons. Post-transformation, the bacteria were eliminated from tissues by a 7-week culture on antibiotic-containing medium. Glyphosate was used to select for transformed tissues containing the *epsps* gene construct. This technique using disarmed *Agrobacterium* followed by selection has a 20-year history of safe use and has been used for transformation of a variety of plant species and tissues (Howard et al. 1990).

Data were provided and reviewed by APHIS that demonstrate stable integration and inheritance of the *epsps* gene and its associated regulatory sequences over several breeding generations. Statistical analyses show that glyphosate tolerance is inherited as a dominant trait in typical

Mendelian manner (petition Table V-1, p. 71). Furthermore, APHIS also concluded that data in the petition (Section V, Figures V-8 and V-16) demonstrate that there was no integration into the alfalfa genome of other protein coding sequences or other genetic elements located outside of the T-DNA borders in the *Agrobacterium* vector (plasmid PV-MSHT4 described on petition pages 34-36) that was used to introduce the CP4 *epsps* gene cassette. There was no unintended change in the genome of alfalfa J101 and J163 as a result of the insertion. The CP4 EPSPS protein is the only new protein expected to be expressed as a result of the transformation process. APHIS evaluated data submitted in the petition (Section V. C - D) on the physicochemical and functional properties of the CP4 EPSPS protein isolated from alfalfa events J101 and J163 that establish its chemical and functional equivalence to the *E. coli*-produced CP4 EPSPS protein used in previous safety studies.

Potential impacts on the relative weediness and/or invasiveness of alfalfa events J101 and J163

APHIS assessed whether alfalfa lines J101 and J163 are any more likely to become weeds than the non-transgenic control populations, or other currently cultivated alfalfa. The assessment encompasses a thorough consideration of the basic biology of alfalfa and an evaluation of unique characteristics of alfalfa lines J101 and J163.

Almost all definitions of weediness stress as core attributes the undesirable nature of weeds from the point of view of humans; from this core, individual definitions differ in approach and emphasis (Baker, 1965; de Wet and Harlan, 1975; Muenscher, 1980; Booth et al., 2003). The parent plant in this petition, *Medicago sativa* L., is not listed as a serious weed in A *Geographical Atlas of World Weeds* (Holms et al., 1991) or as a weed in *World Weeds: Natural Histories and Distribution* (Holms et al., 1997), *Weeds of the North Central States* (University of Illinois, 1988), *Weeds of the Northeast* (Uva et al.,1997), or *Weeds of the West* (Whitson et al., 1992). Alfalfa is not listed as a noxious weed species by the U.S. Federal Government (7 CFR Part 360), is not listed as a weed in the major weed references (Crockett, 1977; Holm, Pancho et al., 1979; Muenscher, 1980), nor is it on other weed lists such as: Washington State Weed Lists (Washington State, 2010), California Weed Species Lists (State of California,1999), Montana County Noxious Weed List (Montana, 2010), and North Dakota Noxious Weeds (Lym, 2004).

Alfalfa is not considered a serious weed, a noxious weed or an invasive species in the United States, even though feral (free-living) populations are fairly common, and volunteers may occur in succeeding crops.

The Southern Weed Science Society (SWSS), in collaboration with the North Central Weed Science Society, Interactive Encyclopedia of North American Weeds Version 3.0 (447 entries), does include alfalfa (NCWSS, 2005). The documentation provided by this list does not indicate why alfalfa is considered a weed. It is possible that it is included because it can be an unwanted volunteer in agricultural settings.

The author of the alfalfa segment of the SWSS Weed Identification guide has stated that alfalfa is not “an invasive weed” nor does it “displace native species,” but alfalfa does colonize disturbed areas (Brett Serviss, Docket No. 04-085-1 #480).

Generally, feral populations (e.g. those that arise as escapes from cultivated fields or from equipment, or from seed spills or contaminants in planted seed), many of which are along roadsides, are not a problem, and generally no attempts are made to control these populations. In some instances, these feral populations are considered advantageous and are encouraged (petition Appendix 3, p. 375, 12/31/02 Letter from South Dakota State University).

Because Roundup Ready commercial alfalfa varieties developed from events J101 and J163 are glyphosate tolerant, herbicides that contain this active ingredient would no longer be effective for control or removal of volunteer or feral GT alfalfa plants or for removal of GT alfalfa stands (stand take-out) when rotating to other alfalfa varieties or other crops unless the herbicide contains additional active ingredients that are effective to control the GT alfalfa lines. Glyphosate is the primary tool used to remove conventional alfalfa stands, therefore, a major difference between GT alfalfa and conventional alfalfa cultivation will be the use of herbicides other than glyphosate for removal of GT alfalfa during stand take-out. Non-glyphosate herbicides and tillage are recommended for effective GT alfalfa stand removal. Several other herbicides, besides glyphosate, are registered, that are effective for stand take-out or conversion and for control of volunteer or feral alfalfa for a variety of different rotational crops or non-crop situations. These same herbicides would still be effective for GT alfalfa. At least eleven other herbicides and mixtures of those herbicides are available to control volunteer GT alfalfa. These are the same herbicides that are used to control non-GT alfalfa with the exception that glyphosate can be used to control non-GT alfalfa. These control options, restrictions, and effectiveness in controlling alfalfa are described in the technical report in the FEIS Appendix J: *Effects of Changes in Farming Practices on Water, Soil and Air Due to Use of Glyphosate-Tolerant Alfalfa*, in particular in Tables J-11 and J-12. Farmers are not able to use glyphosate to control volunteer GT alfalfa in other GT crops. Tilling, mulching and other approved methods can be used for control of volunteer alfalfa in organic farming operations. Feral alfalfa is discussed in more depth in the technical report *Effects of Glyphosate-Tolerant Weeds in Non-agricultural Ecosystems* (Appendix H). Mechanical removal, spot burning, and alternative herbicides can be used to control feral alfalfa depending on the setting.

Alfalfa possesses few of the notable characteristics of successful weeds (Baker, 1965; Keeler, 1989; Booth et al., 2003). APHIS reviewed various data sets comparing J101 and J163 populations and non-transgenic control populations (including also commercially available reference alfalfa varieties) (petition Section VI pages 99-254) to determine whether the J101 and J163 populations were changed in any manner that might affect the plants ability to persist or compete as a weed and to determine the occurrence of unintended effects. Data were reviewed on seed dormancy, seed germination, seedling emergence, seedling vigor, spring stand, spring vigor, seed yield, vegetative growth or plant vigor, plant dormancy, growth habit, flowering properties, effect on symbiotic organisms, as all of these are characteristics that might relate to or have an effect on increased weediness. Observations were also made of response to biotic and abiotic stresses. Data for these phenotypic characteristics (with the exception of the seed dormancy and germination, flowering properties, and nodulation with nitrogen-fixing bacteria) were collected from one or more of a series of three phenotypic studies involving field trials of J101 and J163 in multiple locations.

Different populations of transgenic plants used in the studies have different frequencies of the transgene conferring GT. As explained in the petition (pp. 19-20 and 99 – 100) Lines (events) J101 and J163 will be combined through conventional breeding to produce commercial Roundup Ready alfalfa seed. Commercial Roundup Ready alfalfa varieties will consist of a segregating population of individuals with the *cp4 epsps* gene insert copy number ranging from zero to eight, contributed by either events J101 or J163 or both. A description of the distribution of the *cp4 epsps* gene copy number in a Roundup Ready alfalfa population is presented in Appendix 1 of the petition. Plants with seven to eight copies are extremely rare, and the majority of plants will contain two copies of the *cp4 epsps* gene. Those lacking a copy of the *cp4 epsps* gene are called null segregants and will be susceptible to glyphosate. In the commercial variety (Syn 3 population), approximately 95% of the individuals are expected to be glyphosate tolerant due to the presence of one or more copies of the transgene, and null segregants are expected to comprise about 5% of the individuals (petition Appendix 1, Table 1). The copy number variation is due to the genetics associated with alfalfa breeding and because varieties are comprised of a heterogeneous population of individuals. The petition Figure VI-8 shows the breeding history of populations of J101, J163 and J101XJ163 used in the studies and the percent of each population that are GT (RR= Roundup Ready) vs. not tolerant (NT). The percent of individuals in the single event populations are lower (~50 – 88 percent) compared to the J101XJ163 populations (greater than 90 percent). The phenotypic segregation data for the GT trait in different generations of J101 and J163 are also shown in the petition Appendix 1, Table 2. The petitioners indicate (petition pp. 99-100) that the primary objective of these phenotypic studies was to compare phenotypic parameters of alfalfa plants containing the transgene from either J101 or J163 with these same parameters in control and reference alfalfa plants that lack the transgene. Data derived from single event plants and appropriate control or reference varieties served as the foundation upon which the risk assessment was based. However, data were also developed for the more complex synthetic generations of plants containing both events J101 and J163 (produced through random intercrossing via open pollination of all parent plants) and are presented as confirmatory information to support the conclusions generated from single event populations. These are referred to as the paired J101xJ163 confirmatory populations.

Variations in these the phenotypic studies, with respect to the J101 and J163 populations tested, the locations, the conditions of the trials including herbicide applications, and the phenotypic characteristics evaluated, are summarized below.

This summary is followed by APHIS' analysis of the data and observations included in the petition as it relates to weediness potential.

Phenotypic Study One was conducted from Fall 2001 through Fall 2003 at four locations in California, Iowa, Illinois, and Wisconsin with J101, J163, and the paired J101XJ163 confirmatory alfalfa populations (all segregating for the glyphosate tolerance trait) to evaluate phenotypic equivalency (including measurements of emergence and vigor, crop growth stage at cutting, forage yield, regrowth after cutting, fall plant height and growth habit) (petition Tables VI-10 – VI-14). Each field was periodically rated for abiotic and biotic stressors (e.g., insects, diseases, weeds) and the test, control and reference populations responses to those stresses were noted (petition Table VI-16). Agronomic practices used to prepare and maintain each field site were characteristic of each respective region, but glyphosate was not applied to any of the plots.

Starting material was inoculated with the symbiotic nitrogen fixing bacterium, *Sinorhizobium meliloti*.

Phenotypic Study Two alfalfa field trials were established in 2001 at six locations that represent a range of typical environmental and agronomic conditions for commercial alfalfa production located in the states of California, Iowa, Illinois, New York, Washington, and Wisconsin. The test material consisted of alfalfa populations containing event J101, J163, and the paired J101XJ163; however, unlike Study One, the null segregants were removed from these populations. Only the field plots with the glyphosate tolerant alfalfa populations were treated with the proposed label rates for glyphosate (1.5 lb per acre of glyphosate acid equivalence (a.e.) at up to 4 applications throughout year 1 and no more than 3 applications in year 2). The plots of control or reference varieties lacking the GT transgene were hand-weeded or treated with unspecified herbicides registered for use in alfalfa. Although the primary purpose was to generate test, control and reference substances for use in subsequent product characterization studies, phenotypic observations were also taken to document the condition of the plants from which forage samples were obtained for compositional analysis, and were included as supplemental data to support the conclusions made from Phenotypic Study One. Data were taken on all plots during 2001 and 2002 for general growth and vigor (petition Table VI-17), and insect and disease susceptibility (damage) (petition Table VI-18) using a numerical scale; in 2002, however, insect and disease susceptibility was rated on a qualitative basis (petition Table VI-19).

Phenotypic Study Three involved a 40 month field trial from 1999-2002 in West Salem, Wisconsin that evaluated forage yield (petition Table VI-21), survival (petition Table VI-22), vegetative vigor (petition Table VI-23), fall dormancy reaction (petition Table VI-24), and pest and environmental stress tolerance. J101 and J163 test populations had null segregants removed by treatment with 3.0 lb a.e. glyphosate per acre 16 days after planting, and only tolerant plants were transplanted to the field. Weeds were controlled throughout the trial by mechanical cultivation, and the plots were managed according to locally recommended practices for optimum alfalfa forage production (e.g., fertility, insect pest control, harvest schedule, etc.). Insecticides were applied three times during mid-summer to control potato leafhoppers (*Emposca fabae*).

Seed dormancy and germination characteristics for events J101 and J163 were evaluated in three separate growth chamber experiments from seed produced by FGI in Canyon County, Idaho. Experiments one and two evaluated seed harvested in 2001 and 2002, respectively, from identical plots, and seed germination was evaluated using eight different temperature regimes. A third experiment was conducted to evaluate the percentage of hard seed of potential Roundup Ready alfalfa commercial variety seed lots containing alfalfa events J101 and J163 that were produced in 2003 in Ada and Canyon counties, Idaho. All of the J101 and J163 populations and commercial J101xJ163 paired populations were segregating for the transgene (see petition Figure VI-8).

<u>Seed Dormancy</u>: Seed dormancy, which includes hard seed (a water impermeable seed coat (AOSA, 2002)) as found in alfalfa, has a potential effect on weediness by affecting the soil seed

bank (the total quantity of viable seeds of various species in the soil at any one time) and allowing the seed to remain viable over various seasons and potentially over several years. The percentage of hard seed in alfalfa can vary in a particular seed lot, depending on genetic factors, environmental conditions during and after seed maturation, and harvesting methods (Bass et al., 1988). The data found in the petition for hard seed (petition Section VI pages 114-135) is also quite variable. In the first year, seed of J101 and J163 populations had a significantly higher level of hard seed than the control populations. In the second year, the seed in J101 and J163 populations had a significantly lower level of hard seed than the control populations. In the third year, the seed of ten J101XJ163 populations had both higher and lower levels of hard seed than the ten control populations tested with the mean and the range of the J101X J163 populations being essentially equal to the control populations. In addition, during the public comment period in late 2004 and early 2005 after notifying the public of the availability of the petition 04-110-01p for deregulation and the corresponding environmental assessment, six scientists from four different states, each with more than 20 years experience working with alfalfa and or seed physiology, commented that there was no evidence linking the increased levels of hard seed with glyphosate tolerance. They also generally noted that hard seed in the case of alfalfa was not linked to dormancy since their observations and studies in the past showed that hard seed almost always germinated within a few weeks of seeding and the seedlings that developed were generally too weak and noncompetitive to survive. From the data submitted in the petition and from the comments from these scientists, APHIS concludes that glyphosate tolerance does not cause increased seed dormancy.

Seed Germination: The level of alfalfa seed germination as determined under laboratory conditions is generally negatively correlated to the level of hard seed. Both are determined in the same seed test (AOSA 2002). If the percentage of hard seed is determined to be high in the seed test, then the percentage of seed germination is generally lower. If the percentage of hard seed is low, then the percentage of seed germination is generally higher. Increased levels of seed germination may have an effect on weediness by allowing more plants to establish in the environment by outnumbering plants with lower levels of germination. The data in the petition for seed germination (petition Section VI pages 114-135) are best explained by the levels of hard seed in the various samples of seed tested for each of the populations of J101, J163, J101XJ163, control, and representative varieties. The overall mean percentage and range of normal germinated seed for ten J101XJ163 test populations which contained 95 percent of GT individuals was comparable to the nontransgenic control populations which shared a common ancestry (petition Table VI-6). From the data submitted in the petition, APHIS concludes that glyphosate tolerance does not cause increased seed germination.

Seedling Emergence: Seedling emergence is a combination of seed germination rate, seed dormancy which may decrease the number of seeds germinating, and the effect of various environmental conditions on the germination process such as cold soils, water availability, insect predation, and level of disease pressure. Increased seedling emergence may have an effect on weediness by allowing more plants to establish in the environment by outnumbering plants with lower levels of seedling emergence. Therefore seedling emergence is an overall score for the level of plant survival between seeds planted and surviving seedlings. The seedling emergence data in the petition (petition Section VI pages 135-157) indicate that seeds of J101 and J163 populations performed essentially the same as the control populations or reference varieties.

From the data submitted in the petition, APHIS concludes that glyphosate tolerance does not cause increased seedling emergence.

Seedling Vigor: Increased seedling vigor may have an effect on weediness by allowing more seedling plants to establish in the environment by outnumbering and outcompeting plants with lower levels of seedling vigor. Increased seedling vigor is a combination of early seedling establishment, increased vigorous seedling growth, and tolerance to seedling diseases. The seedling vigor data in the petition (petition Section VI pages 135-157) indicate that seeds of J101 and J163 populations had essentially the same vigor as seeds of the control populations or reference varieties. From the data submitted in the petition, APHIS concludes that glyphosate tolerance does not cause increased seedling vigor.

Spring Stand: This is a rating for winter survival or winter hardiness. Higher ratings for spring stand would indicate better winter survival. Increased spring stand may have an effect on weediness by outcompeting other more desirable plants, and allowing more plants to survive and compete in the environment to the detriment of other plants. The spring stand data in the petition (petition Section VI, pages 135-157) indicate that J101 and J163 populations had essentially the same rating for spring stand as the control populations or reference varieties. From the data submitted in the petition, APHIS concludes that glyphosate tolerance does not cause increased spring stand.

Spring Vigor: This trait could be considered a combination of spring stand and the rate of plant growth in the spring. A dense spring stand along with rapid spring plant growth would be very detrimental for the growth of other plant species. Dense alfalfa stands are known to be very competitive against weeds. On the other hand the argument could be made that an alfalfa variety that has an increased ability to provide a denser spring stand along with excellent spring vigor could be considered weedier if allowed to grow in an area where it is not wanted. The spring vigor data in the petition (petition Section VI pages 135-157) indicate that J101 and J163 populations had essentially the same spring vigor as the control populations or reference varieties. From the data submitted in the petition, APHIS concludes that glyphosate tolerance does not cause increased spring vigor.

Seed Yield: High seed yield is a desirable characteristic of a successful commercial alfalfa variety since seed yield is a major component in the final selling price. Higher seed yields (weight per acre) generally translate into lower selling prices if all other factors are held constant. On the other hand, the argument could be made that an alfalfa variety that has a higher seed yield could be considered weedier if allowed to grow in an area where it is not wanted. Higher seed yield would mean more seeds and or bigger seeds would be produced that could give it a selective advantage. The seed yield data in the petition provided information on seed weight per 1000 pollinations, seed weight per plant, weight per seed, and the number of seeds per flower (petition Section VI pages 209-217) for the J101 and J163 populations and the control populations or reference varieties. From the data submitted in the petition, APHIS concludes that glyphosate tolerance does not cause increased seed yield.

Vegetative Growth or Plant Vigor: High forage yield is a desirable characteristic of a successful alfalfa variety since a high yield of highly nutritious forage is the goal of forage production for

animal consumption. Good plant vigor is also necessary for minimizing weed competition in a forage field. More vegetative growth or more plant vigor could also be considered a characteristic that could lead to more weediness. The petition provided information on vegetative growth or plant vigor in the form of forage yield (fresh weight per acre or weight per plant), crop growth stage (Mean Stage by Count), regrowth after cutting, and growth and or vigor scores (petition Section VI pages 135-137, 167-170, 175-182) , for the J101 and J163 populations and the control populations or reference varieties. A significantly higher forage yield for one or more cuttings or total weight for the year could possibly indicate a faster growing or larger plant that in the right environment could indicate more weediness. A significantly higher crop stage growth rating would indicate that more plants could be producing seeds more rapidly which also could mean more weediness. A significantly higher rating for regrowth after cutting could possibly indicate that the plants are more vigorous after cutting or grazing, possibly making these plants more competitive against other plants. Growth and or vigor scores that are consistently and significantly higher over dates within a year, over years, and over locations, could also possibly provide information on the potential competitiveness of a plant which may make it weedier. From the data submitted in the petition, no significant consistent pattern was noted for higher forage yield, higher crop stage growth rating, higher regrowth rating, or higher growth and vigor scores. Thus APHIS concludes that glyphosate tolerance does not cause increased vegetative growth or plant vigor.

Plant Dormancy: This characteristic and its relationship to possible weediness is highly dependent on the climate in which the plant is grown. More plant dormancy is critical for a perennial plant's survival in climates with freezing winter temperatures. A plant that is actively growing when freezing temperatures occur will generally die. On the other hand, if the plant has a high level of dormancy going into the fall of the year (the plant slows or stops active growth and the various physiological properties are conditioning the plant for withstanding freezing temperatures), and the plant is growing in a climate with no freezing winter temperatures, the plant is at a competitive disadvantage to those plants that are actively growing. Alfalfa varieties vary widely in dormancy from no hardiness to high levels of dormancy. In the petition, fall dormancy was determined by plant height measured in the fall after the last cutting of the season or as a visual rating. Greater fall plant height and higher visual ratings would indicate greater fall dormancy. From the data submitted in the petition (petition Section VI pages 135-157, 175-182), no significant differences were noted for fall plant height or visual fall dormancy ratings between J101 and J163 populations and the control populations or reference varieties. Thus, APHIS concludes that glyphosate tolerance does not change fall dormancy.

Growth Habit: A prostrate growth habit or an upright growth habit does not necessarily impart a weediness characteristic to a plant species or variety; the effect may depend more on the relationship of the plant to the plant community in which it is growing. A rapidly growing plant with a prostrate growth habit would be more competitive against plants that are slow to germinate or slow to regrow in the spring or after grazing. A rapidly growing plant with an upright growth habit would be more competitive in a denser stand of plants slow to germinate or slow to regrow in the spring or after grazing. From the data submitted in the petition (petition Section VI pages 135-137, 158), no significant differences were noted between J101 and J163 populations and the control populations or reference varieties in fall growth habit. Therefore, APHIS concludes that glyphosate tolerance does not change fall growth habit.

Survival: For a perennial crop, survival is an important characteristic since it helps determine the economically viable time length of commercial fields. If the alfalfa forage fields can survive longer, the significant costs involved with establishing and destroying fields can be avoided for longer periods of time and prorated over more harvests. It is a measure of a variety's tolerance of various biotic and abiotic stresses, such as frequent mowing/grazing, freezing temperatures, diseases, insects, traffic from vehicles and animals, etc. The possibility exists that if an alfalfa plant can survive longer when stressed by the same biotic and abiotic factors, then it may have a greater potential to become weedy in some circumstances. From the data submitted in the petition (petition Section VI pages 175-182), no significant differences were noted between J101 and J163 populations and the control populations or reference varieties in survival during the 40 month trial, and APHIS concludes that glyphosate tolerance does not change survival.

Flower and Pollen Morphology: For alfalfa that is dependent on bees for cross pollination and seed production, changes in flower morphology may have an effect on bee visitation. Exactly how changes in flower morphology may affect weediness is not understood, so if any changes are noted then it needs to be examined more closely. Data submitted in the petition (petition Section VI pages 183-209) provided measurements on number of flowers per raceme, length of the standard petal, length of the keel petal, diameter of the calyx tube, length of the sexual column, number of pollen grains per flower, percent pollen germination, percent pollen viability, pollen diameter, and visual observation of the following: flower color, general flower morphology, rachis attachment to the stem, flower attachment to the rachis, flower ripening pattern of the raceme, general observation of the pollen load per flower, self fertility of flowers whether tripped without any assistance or mechanically tripped by the researcher, and general seed morphology. From these data submitted in the petition, no significant differences between J101 and J163 populations and the control populations or reference varieties were noted for any of these flower and pollen characteristics. Therefore, APHIS concludes that glyphosate tolerance does not change flower and pollen characteristics, and therefore cannot have an effect on weediness.

Symbiotic Organisms: Since nitrogen-fixing bacteria (*Sinorhizobium meliloti*, formerly known as *Rhizobium meliloti)* are known to have a symbiotic relationship with legumes, data and observations were taken regarding nodule formation on inoculated greenhouse grown seedlings (petition Table VI-39 and Figure VI-22) and on one year old plants from a field with sufficient levels of existing inoculum (petition Table VI-23) (petition Section VI pages 248-251. In addition to nodule formation, other phenotypic characteristics were also examined that would be affected by modification of the nitrogen fixing process, such as seedling growth in the inoculated greenhouse grown plants (petition Table VI-39, Section VI pages 248-251); and levels of total protein (as derived from total nitrogen), asparagine, and aspartate in alfalfa forage (petition Table VI-34, Section VI pages 223-247), and forage yield (petition Section VI pages 135-137 and 175-182) from field grown plants. Reported effects of glyphosate on microbial interactions with crops (including those engineered for resistance to glyphosate) are addressed in the FEIS in Chapters 3 and 4. Glyphosate applications were used in some of the studies, but none of the studies were specifically designed so as to detect effects specifically related to glyphosate use, since the focus of the assessment was on the effects of the genetic transformation. APHIS did consider differences in the way the studies were conducted with respect to glyphosate applications as well as inoculation with, or availability of, nitrogen-fixing bacteria. Mature plant

nodule observations and forage compositional analysis were performed on plants in which the field plots (with the exception of the control plots) had been treated with glyphosate, and the plants were not inoculated with *S. meliloti*. No biologically meaningful differences were detected between J101, J163 or J101XJ163 populations and control or reference populations. No glyphosate treatments were applied in the seedling studies or forage yield studies. Forage yield data were collected from Phenotypic Studies One and Three (described above), only the first of which was inoculated with *S. meliloti*. From these data submitted in the petition, and regardless of the differences in the inoculum status and glyphosate treatments between these studies, no significant or biologically meaningful differences were noted between the J101 and J163 populations and the control populations or reference varieties for any of these symbiotic nitrogen fixing relationships. Thus, APHIS concludes that the glyphosate tolerance trait does not affect symbiotic relationships that have a bearing on the parameters measured, and therefore no increase in weediness is expected from enhanced symbiotic relationships.

No unusual characteristics were noted that would suggest increased weediness of J101 and J163 plant populations. Additionally, no characteristics relating to disease or insect resistance that might affect weediness were noted. These characteristics were consistent over all field trial locations, some of which were treated with glyphosate and some which were not (petition Tables VI-16, VI-18, VI-19, VI-20 pages 159-175). J101 and J163 alfalfa are still susceptible to the typical insect and disease pests of alfalfa. Therefore, there is no selective advantage to alfalfa containing CP4-EPSPS compared to conventional alfalfa, and there is no increased potential for weediness or invasiveness from alfalfa events J101 and J163.

Potential impacts from gene flow and gene introgression from alfalfa events J101 and J163 into sexually-compatible relatives

APHIS evaluated the potential for hybridization and gene introgression to occur from alfalfa events J101 and J163 to sexually compatible wild (free-living) relatives, and considered whether such introgression would result in increased weediness. Alfalfa is sexually compatible with several subspecies within the *M. sativa* complex (Small and Jomphe, 1989). The center of origin for the genus *Medicago* is generally believed to be in the Caucasus, northwestern Iran and northeastern Turkey; the genus is not native in North America. An additional 18 *Medicago* species are known to be naturalized (free-living) or possibly so within the United States, of which only *M. lupulina* (black medic) is widely naturalized throughout the United States. None of these species are native to the United States, and none are sexually compatible with *M. sativa*. The *M. sativa* complex, which was introduced into North America early by Europeans for forage and includes all the commercial alfalfa varieties, is a group of closely related subspecies, including the cultivated *M. sativa* ssp. *sativa* and *M. sativa* ssp. *falcata* (synonym *M. falcata*) (Small and Jomphe, 1989).

In addition to the *M. sativa* complex within which all of the subspecies are sexually compatible to some degree, an additional 17 and possibly 18 *Medicago* species have been recognized as being naturalized (free-living) or possibly so in the United States (USDA-NRCS, 2004; Kartesz, 2004). All of these 18 species are annual species, except for the species *M. hybrida* (in *Medicago* section *Medicago*) hybrids, which have only been produced experimentally by embryo

culture. No annual species are known to hybridize with *M. sativa* (Quiros and Bauchan, 1988; McCoy and Bingham, 1988; and the petition's Appendix 4).

Medicago lupulina (black medic) is the species that might be of most concern within this list of 18 species. It is considered a weed in lawns and waste places and in forages since its seeds frequently contaminate forage legume seed crops. Black medic is an annual (possibly sometimes short-lived perennial) (Tarkington and Cavers, 1979) self-pollinating species and is known to occur throughout the United States. Successful hybridizations between *M. sativa* and *M. lupulina* have been reported (Southworth, 1928; Fryer, 1930; Shrock, 1943). However, because of the lack of hybrids after many subsequent experiments, and the demonstrated genetic differences between these two species, there is general agreement that these putative "hybrids" were most likely not hybrids but were the result of self-fertilization (Lesins and Gillies, 1972; Fridriksson and Bolton, 1963; Valizadeh et al., 1996). For more details on this topic, see Section E.1 (p. 284) and Appendix 4 of the petition. Based on all the recent data available on this subject, APHIS' opinion is that hybridization between *Medicago sativa* and *M. lupulina* has an extremely low to non-existent probability of occurring in a non-experimental or even in an experimental setting.

APHIS concludes that the potential of the glyphosate tolerance trait to move from J101 and J163 to other sexually compatible *Medicago* species in the United States is essentially non-existent.

Potential impact of alfalfa events J101 and J163 on disease and pest susceptibilities

The data submitted by Monsanto and FGI indicated no significant differences between alfalfa J101 and J163 populations and the nontransgenic control populations or reference varieties for disease observations (as measured by seedling damping-off (e.g., fungal genera such as *Pythium, Phytophthora, Aphanomyces*); foliar diseases (e.g., fungal genera such as *Leptosphaerulina, Colletotrichum, Peronospora, Phoma, Stemphylium, and Cercospora);* stem nematodes like *Ditylenchus*; root rots, vascular wilts and crown diseases (e.g., fungal genera such as *Phytophthora, Verticillium, Fusarium, Phoma,* and bacterial wilt caused by *Clavibacter*); pest susceptibility (as measured by potato leafhoppers (*Empoasca fabae*), aphids [pea (*Acyrthosiphon pisum*), blue (*A. kondoi*) and spotted alfalfa aphids (*Therioaphis maculata*)], alfalfa weevil (*Hypera postica*), lygus bugs (*Lygus* species), other plant bug species (family *Miridae*) and alfalfa caterpillars (various lepidopteran species)) (Tables VI-16, VI-18 to VI-20 of petition pages 159-175). In Phenotypic Study One (described above) disease pressure was low for most observation periods for the four locations and no differences were noted between test, control and reference alfalfa lines for those diseases that were detected (Table VI-16). In Phenotypic Study Two, analysis of variance testing was conducted on data collected regarding growth and vigor, insect damage and disease damage pooled across sites, years, and observations (Table VI-20) and indicated no significant differences between glyphosate-treated, glyphosate tolerant alfalfa versus non-glyphosate-treated control alfalfa. There were no consistent trends that would indicate increased susceptibility to stressors that frequent alfalfa fields, although few stressors were observed during the course of the trials in this study. There were, however, incidences of drought and water stress which could conceivably change the level of disease pressure; but few diseases were noted (only Black mold and Leaf Spot). Overall disease rating scores for all

treatments, locations, and dates were high in 2001 indicating that few disease symptoms were observed. Mean ratings ranged from 4.0 to 5.0 on a scale from 1 to 5, with 1 indicating 76-100 percent of plants with symptoms and 5 indicating 0-5 percent of plants with symptoms.

The data presented in the petition also indicates no difference in compositional and nutritional quality of glyphosate-treated alfalfa events J101 and J163 compared to non-glyphosate treated conventional alfalfa, apart from the presence of CP4-EPSPS. Although some of the variables measured by the applicant showed statistically significant differences between alfalfa events J101 and J163 and the nontransgenic control population (Tables VI-34 and VI-35 of the petition, pages 226-244), none of the values for the forage composition characteristics were outside the range of natural variability of conventional alfalfa as found in the literature (Table VI-36 of the petition, page 245). Therefore, the composition of forage from J101 and J163 alfalfa is not biologically different than conventional alfalfa, which also supports that they would be susceptible to the same pest populations as conventional alfalfa. Additionally, J101 and J163 alfalfa events, whether sprayed with glyphosate or not, were found to be similarly affected by typical plant diseases found in alfalfa, and do not harbor an altered pest or pathogen community compared to other alfalfa varieties. Therefore, pest and disease control methods are expected to be similar, and no direct or indirect plant pest effect on raw or processed plant commodity is expected.

Potential impacts from transferring genetic information from alfalfa J101 and J163 to organisms with which it cannot interbreed

APHIS examined whether horizontal gene transfer (HGT) between alfalfa events lines J101 and J163 is likely to occur and whether such HGT would lead to an increased plant pest risk, e.g., through the creation or alteration of microorganisms, pests, or parasites that cause disease or damage to plants. HGT between unrelated organisms is one of the most intensively studied fields since 1940, and the issue gained extra attention with the release of transgenic plants into the environment (Dröge et al. 1998). HGT has been implicated as a major contributor to the spread of antibiotic resistance amongst pathogenic bacteria and the emergence of increased virulence in bacteria, eukaryotes, and viruses; and has contributed to major transitions in evolution. Gene exchange has been documented for nearly all types of genes and between unrelated organisms (Gogarten et al. 2002).

Horizontal gene transfer and expression of DNA from a plant species to other bacterial species is unlikely to occur based on the following observations. Although there are many opportunites for plants to directly interact with fungi and bacteria (e.g. as commensals, symbionts, parasites, pathogens, decomposers, or in the guts of herbivores), there are almost no evolutionary examples of HGT to bacteria from eukaryotes or from plants to fungi (as reviewed in Keese 2008). Accumulated evidence show that there are universal gene-transfer barriers, regardless of whether transfer occurs among closely or distantly related organisms (Kaneko et al., 2000; Koonin et al., 2001; Wood et al., 2001; Kaneko et al., 2002; Brown, 2003; Sorek et al., 2007). Many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Kaneko et al., 2000; Kaneko et al. 2002; Wood et al., 2001). There is no evidence that these organisms contain genes derived from plants. In cases where review of sequence data implied that horizontal gene transfer occurred, these events are

inferred to occur on an evolutionary time scale on the order of millions of years (Koonin et al., 2001; Brown 2003). Recently, Yoshida and colleagues (Yoshida et al., 2010) through a comparative genomics analysis implicated HGT for the presence of a similar genetic sequence between the parasitic plant purple witchweed (*Striga hermonthica*), which infests cereal fields (monocots), and sorghum (*Sorghum bicolor*). According to the authors, the incorporation of a specific genetic sequence occurred between sorghum and purple witchweed before speciation of purple witchweed (*S. hermonthica*) and related cowpea witchweed (*S. gesnerioides*), a parasitic plant of dicots, from their common ancestor. In other words, HGT is an extremely rare event, and a majority of those rare events occur over millions of years.

With respect to the specific genetic sequences inserted into J101 and J163 alfalfa, microorganisms and plants already contain genes that encode EPSPS enzymes. The evolutionary origins of enzymes in the shikimate pathway was recently summarized by Richards et al. (2006), who indicate that the shikimate pathway is reported as a metabolic feature of plants as well as prokaryotes, and has been retained in many eukaryote lineages, now known to comprise the zygomycete, basidiomycete, and ascomycete fungi, apicomplexa, ciliates, and the oomycetes. Thus, the unlikely HGT of the EPSPS gene from the J101 and J163 to these microorganisms is unlikely to change their pathogenicity status.

Furthermore, transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. HGT from *Agrobacterium* species, such as the CP4 strain from which the sequence inserted into J101 and J163 was derived, to other bacteria would be more likely to occur and result in gene expression than would HGT from plants. Thus even if horizontal gene transfer from events J101 and J163 occurred to bacteria, proteins corresponding to the transgenes are not likely to be produced.

APHIS also considered whether HGT of the Figwort mosaic virus 35S promoter inserted into events J101 and J163 to plant viruses was likely to occur, and would lead to the creation or selection of a more virulent plant pathogen through recombination with other plant viruses. HGT to plant viruses has been considered before by other science review panels and government regulatory bodies (for a general review of the issue see Keese 2008 and GM Science Review Panel 2003). The only potential for a plant virus promoter already integrated into a plant genome to recombine with another plant virus is through integration or recombination of the plant virus in the plant's genomic DNA. According to a recent review of viral sequences integrated into plant genomes (Harper et al. 2002), "As with animal and bacterial viruses, it is only those viruses with DNA genomes or those that have a DNA phase in their replication that have integrated sequences. In spite of the majority of plant viruses having RNA genomes, there are no examples yet of integrated forms of these viruses." The figwort mosaic virus is a Caulimovirus that consists of double stranded DNA, and *Medicago* species are not considered susceptible hosts of the Caulimoviruses (Plant Viruses Online – Brunt et al. (eds.) 1996 onwards). Therefore recombination of the Figwort mosaic virus 35S promoter with caulimoviruses is unlikely to occur, and recombination would also be unlikely with more distantly related viruses with a DNA phase that affect alfalfa, such as geminiviruses and nanaviruses (Plant Viruses Online – Brunt et al. (eds.) 1996 onwards). None of the noncoding regulatory regions are expected to have any plant pest effect even if HGT were to occur.

Therefore APHIS concludes that horizontal gene transfer is unlikely to occur and is unlikely to pose a plant pest risk.

Conclusion

APHIS has reviewed and conducted a plant pest risk assessment on alfalfa events J101 and J163 alfalfa. Based on APHIS's analysis of field, greenhouse, and laboratory data and references provided in the petition and other relevant information as cited, APHIS concludes that alfalfa events J101 and J163 are unlikely to pose a plant pest risk for the following reasons. (1) They exhibit no plant pathogenic properties – although a plant pathogen was used in their development, these plants are not infected by this organism nor do they contain genetic material from pathogens used as a donor organism that can cause plant disease. No new protein other than the intended CP4 EPSPS was produced and there was no unintended change in the genome of alfalfa J101 and J163 as a result of the insertion. (2) They exhibit no characteristics that would cause them to be weedier than the non-transgenic parent alfalfa or other cultivated alfalfa and several control options besides glyphosate are available for control of feral or volunteer plants or for stand take-out. (3) Gene introgression from J101 and J163 to native, introduced, or naturalized species of *Medicago* in the United States is extremely unlikely and even if it were to occur, is not likely to increase the weediness potential of any resulting progeny any more than would introgression from other cultivated alfalfa. (4) Disease and insect susceptibility and compositional profiles of J101 and J163 are similar to those of the parent variety and other alfalfa cultivars grown in the United States, therefore pest and disease control methods are expected to be similar and no direct or indirect plant pest effect on raw or processed plant commodity is expected. (5) Field observations, compositional data including components related to nitrogen fixation and nodulation, and data on the safety of the engineered EPSPS protein all indicate that J101 and J163 should not have a greater potential than other cultivated alfalfa to damage or harm organisms beneficial to agriculture. (6) Horizontal gene transfer from these alfalfa events is highly unlikely to occur, and is not expected to pose a plant pest risk.

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