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Review

Considerations for Managing Agricultural Co-Existence between Transgenic and Non-Transgenic Cultivars of Outcrossing Perennial Forage Plants in Dairy Pastures

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Abstract: Many of the major forage species used in agriculture are outcrossing and rely on the exchange of pollen between individuals for reproduction; this includes the major species used for dairy production in grazing systems: perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Cultivars of these species have been co-existing since contrasting cultivars were developed using plant breeding, but the consequences and need for strategies to manage co-existence have been made more prominent with the advent of genetic modification. Recent technological developments have seen the experimental evaluation of genetically modified (GM) white clover and perennial ryegrass, although there is no current commercial growing of GM cultivars of these species. Co-existence frameworks already exist for two major cross-pollinated grain crops (canola and maize) in Europe, and for alfalfa (*Medicago sativa* L.) in the US, so many of the principles that the industry has developed for co-existence in these crops such as detection techniques, segregation, and agronomic management provide lessons and guidelines for outcrossing forage species, that are discussed in this paper.

Keywords: pasture; GMO; co-existence

1. Introduction

Many of the major forage species used in agriculture are outcrossing and rely on the exchange of pollen between individuals for reproduction. This includes the major species used for dairy production in grazing systems: perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Cultivars of these species have been co-existing since contrasting cultivars were developed using plant breeding, but the consequences and the need for strategies to manage co-existence have been made more prominent with the advent of genetic modification.

Genetically modified (GM) crops have been grown commercially for more than 20 years, with more than 170 million hectares sown across 28 countries in 2015 [1]. The majority (70%–90%) of these GM crops are used for animal feed [2] with up to 95% of the 9 billion animals grown for food production each year in the USA consuming diets containing GM ingredients [2]. Until recently this consumption was entirely based on the use of grains (soy, maize) or crop residues (cottonseed meal and canola meal). However, the recent release of Roundup Ready alfalfa (*Medicago sativa*) and research

in other forage crops such as perennial ryegrass and white clover [3,4] suggests that it is timely to consider the co-existence of GM and non-GM pastures.

Co-existence frameworks already exist for two major cross-pollinated grain crops (canola and maize) and alfalfa for seed and hay production, so many of the principles that the industry has developed for co-existence in these crops such as detection techniques, segregation, and agronomic management provide lessons and guidelines for outcrossing forage species.

Some of the principles that underpin a co-existence framework include,

- The ability to detect the transgene or its products in relevant commodities.
- A knowledge of the mechanism and extent of pollen (gene) flow and seed dispersal in the species.
- The strategic use of management interventions to "separate" GM and non-GM crops and prevent gene flow between them.
- The equivalence or otherwise in the agronomic or nutritional aspects of the GM and non-GM crops.
- The segregation of products during marketing and supply.

An example of these co-existence frameworks for grain crops are those developed within the European Union (EU) [5] which allow "the ability of farmers to make a practical choice between conventional, organic, and GM crop production" considering issues such as the segregation of GM and non-GM crops and the cost of this segregation. The principles of such a framework have been adopted by several European nations for the commercial cultivation of GM maize [6]. The technical and social aspects of the co-existence of GM and non-GM maize crops have been reviewed for Spain [7] and the EU [6]. The isolation distances for GM maize proposed by European member states vary from state to state and whether the GM crop is neighbouring a conventional or organic crop with distances varying from 25 to 800 m [8]. A recent review found that the large and fixed isolation distances proposed by some countries were not consistent with either the co-existence principles outlined by the European Commission and were excessive based on scientific evidence [6]; they recommend that isolation distances within the range of 10–50 m would in most instances be sufficient to keep GM inputs from cross-pollination below the legal tolerance level of 0.9%. Therefore, despite inconsistency in the application of the guidelines for co-existence, the principles are well established in grain crops such as maize.

In contrast to grain crops where maximising seed set (through gene flow) is usually the goal, the perennial forage supply chain has two distinct phases,

1. Seed production where high pollination is required
2. Pasture production where most management seeks to minimise reproductive development and seed set—particularly in dairy grazing systems.

In contrast to grain crops where the adoption of GM crops has been widespread, in some countries a range of technical and economic constraints [4] has meant there is only one perennial outcrossing forage crop; glyphosate resistant alfalfa (*Medicago sativa* L.) that was finally approved for fully deregulated, commercial release in the United States (US) in January 2011. The National Alfalfa and Forage Alliance in the US has developed a set of guidelines for the co-existence of alfalfa seed crops [9], and the technical aspects of co-existence and market assurance for alfalfa hay and forage production in an era of biotech crops have been summarized by Putnam et al. [10].

In this paper, we will summarise the literature on aspects of functional equivalence and co-existence in perennial outcrossing forage species with a particular emphasis on those used in dairy production systems drawing on examples using both GM and non-GM plants.

2. Detection of Transgenes in Forages and Related Agricultural Products

Fundamental to the process of monitoring transgenic crops and agricultural commodities is the ability to detect the transgene or its products. Although DNA fragments from high copy number

endogenous plant genes such as rubsico have been detected in the blood and digesta [11,12], transgenic DNA (tDNA) has been shown to be broken down in the rumen and duodenum of cattle [9], and a number of studies have shown that tDNA was not detected in the milk of cows fed diets containing GM feeds [11,13–19]. These data from milking trials are consistent with those from wide ranging reviews of animal production trials that have focused on meat producing animals [2,20] with the conclusion that there are no detectable or reliably quantifiable traces of GM feed components in eggs, meat, or milk [2,20].

Therefore, efforts in GM detection for perennial forages should not focus on milk or meat but rather on other agricultural products such as pollen, seed, and herbage. While the need to detect transgenes in these products themselves is obvious as pollen is the vehicle for gene transfer, seed is traded for sowing new pastures and herbage is the diet of grazing ruminants.

There is also the need to monitor the pollen of perennial forage species that are pollinated by honeybees, as this pollen may find its way into honey. Honey containing traces of pollen from genetically modified plants is currently subject to marketing and labelling regulations in the European Union [21] following the commercial expansion of genetically modified field crops. Studies of the amount of canola pollen occurring in honey from hives foraging non-GM crops in Australia and GM crops in Canada found canola pollen levels on the order of 0.2% and thus well below the 1% threshold for labelling in Australia [22]. White clover pollen is also present in honey produced by bees foraging white clover flowers [23]. The exact cut-off levels for detection are likely to vary from jurisdiction to jurisdiction as well as whether pollen is seen as a constituent or an ingredient of honey [21]. Regardless of these trade and regulatory discussions, the likely presence of genetically modified pollen in honey following commercial release of GM white clover will most likely create a situation analogous to that in canola (e.g., [22]) where commercial honey supplies will need to be monitored.

An example to demonstrate the ability to detect tDNA in a range of products from an insect pollinated perennial forage species comes from research associated with the development and evaluation of white clover with transgenic virus coat protein mediated resistance to Alfalfa Mosaic Virus (AMV) [24]. During the production of genetically modified white clover seed, white clover plants were pollinated by honey bees under containment conditions and PCR-based techniques were developed to detect the AMV coat protein gene and the *neomycin phosphotransferase 2* (*npt2*) selectable marker gene in genetically modified white clover pollen, whether this pollen was collected fresh from honey bees that have been foraging white clover or from honey [25]. Similarly, the AMV coat protein gene was able to be detected in seed, fresh leaves (as would be fed at grazing), air dried leaves (as would be fed as hay) when the leaves were either pure white clover, or in a mixture with perennial ryegrass (simulating the mixed sward systems where white clover is most commonly grown) [26].

These results demonstrate that it is possible to develop molecular diagnostics for pollen, herbage, or seeds from forage plants. The issues of the cost of real-time PCR based systems, which are the standard reference method for transgene detection, when used for routine field-based applications have been addressed in grain crops already through efficient sampling strategies [27–29] and through the development of other diagnostic tools such as semi-quantitative enzyme-linked immunosorbent assay (ELISA) (e.g., [29]) which have also been used to detect the presence of the *CP4 EPSPS* gene in GM bentgrass [30] and alfalfa [10], and through the use of plasmid DNA for the calibrated detection of specific transgenic events (e.g., [31,32]. More recently novel DNA amplification techniques such as recombinase polymerase amplification (RPA) for the rapid point-of-use screening of transgenic soybean seeds [33] have been developed. The ultimate choice of molecular diagnostics for forage samples will depend on the cost and target detection limits, but the experience from major food crops and initial data from forage samples demonstrates that it will be possible to develop these tools for samples from dairy pastures.

Whilst there is no evidence to suggest that tDNA will find its way into the muscles or milk of lactating cows following digestion, the development of methodologies to detect the presence or absence of the *cp4epsps* transgene from soybean meal and the *cry1a*[*b*] transgene from GM corn grain in

the rumen fluid, duodenal digesta, milk, blood, and faeces of lactating cows when fed these diets or their near-isogenic comparators [11] demonstrates that it is possible to monitor the digestion of tDNA in the digestive tract of ruminants.

3. Composition and Performance of GM Feeds and Forages

Although the entry of GM forages into the marketplace is relatively new, the use of GM grain as animal feed has occurred for more than 20 years. Both the experimental studies and the trends following the commercial feeding of GM feed to over 100 billion animals were reviewed by Van Eenennaamm and Young [2] with the conclusion that no study had revealed any difference in the nutritional composition of animals fed GM or non-GM diets nor where there any negative trends in commercial animal health or productivity [2]. Of specific interest to this paper are the results of a 2 year feeding study on the feeding of GM corn (whole-crop silage; kernels and cobs) of GM corn modified with the Bt-MON810 event and an isogenic comparator to dairy cows. This long term study concluded that there were no consistent effects on milk composition or cow body condition and hence the GM corn and its isogenic comparator could be said to have nutritional equivalence, and the milk produced had no functional reason to be classified differently [18,19].

Given the small number of genetically modified perennial forages that have progressed to feed trials, it is not surprising that there are few data sets describing the agronomic or nutritional equivalence of GM cultivars and the non-GM cultivars that the transgenic event has been crossed in to. However, in each of the cases that have been published to date, the data shows no evidence that the performance of the GM cultivars is different to equivalent conventional cultivars other than for the trait controlled by the transgene.

For instance, in a feeding experiment with Holstein cows diets were prepared that were nutritionally similar and contained approximately 40% (by dry matter) of lucerne hay that was either "Roundup Ready" (containing the *cp4epsps* protein) or three conventional cultivars that had been selected to have similar nutritive characteristics to the hay derived from the GM cultivar [17]. In this experiment there were no differences in daily milk yield, fat corrected milk yield, milk fat, milk lactose, non-fat milk solids, nor dry matter intake of cows consuming GM or non-GM diets [17].

In white clover in Australia (where licence conditions prevented the feeding of GM clover herbage to animals), a proximate analysis of both nutritional and anti-nutritional characteristics of the virus coat protein mediated AMV resistant GM white clover was performed [24]. In this study there was no difference in the nutritional characteristics (crude protein, in vitro dry matter digestibility, neutral detergent fibre, and water soluble carbohydrates) and anti-nutritional characteristics (cyanogenic glucosides, phytoestrogens, and saponins) were compared for two conventional white clover cultivars (Mink, Grasslands Sustain) and their GM AMV resistant derivatives when grown under field or glasshouse conditions [34].

A further study on Zoysia grass (*Zoysia japonica* Steud), genetically modified to be resistant to the herbicide glufosinate [35], was shown to be no different to non-GM plants for a range of morphological traits related to turfgrass agronomy and also the allergenicity of the pollen to humans as assessed through skin prick tests [36].

4. Gene Flow in Out-Crossing Perennial Forage Species

The major biosafety concern with cross-pollinated perennial forages is the gene flow from GM to non-GM crops [4]. However, in the case of commodities where the GM and non-GM crops have substantive equivalence with respect to all traits rather than the GM trait, and that the GM trait has been deregulated, the issue is not really one of biosafety per se but rather one of compliance with regulatory guidelines for co-existence. The setting of these thresholds is a matter for commerce and industry; the following discussion describes the aspects of gene flow in perennial forage species during both seed and forage productions and how these data can be effectively used during the design and implementation of a co-existence strategy for forage species.

Perennial forage species may be either wind or insect pollinated. We have shown gene flow in field grown wind pollinated, perennial ryegrass [37], and insect pollinated, white clover [38] follows a leptokurtic distribution with a rapid decline in effective pollen flow such that greater than 95% of gene flow occurs within a relatively short distance of the pollen source (30–50 m) under field conditions. However, a small amount of pollen moves a long distance from the pollen source. These data are consistent with that observed internationally and for major outcrossing grain crops such as maize and canola. These principles have been used to develop isolation distances used during seed production [39].

As the pattern of pollen flow in both wind and insect pollinated species is leptokurtic, it is possible for small amounts of pollen to travel a large distance. If this pollen finds a suitable recipient population it is possible for novel traits (including GM) to establish themselves a long way from the pollen source [30,40,41]. The extent to which these novel traits will establish themselves in the new populations will depend on the reproductive fitness of the plants containing the new trait, the ability of the species to establish new plants through seedling recruitment, and also adaptation of the background genetics of the pollen donor to the new environment.

4.1. Isolation, Separation, and Segregation of Seed Crops

As there is no reason to believe that the pollen of genetically modified forages generally behave differently to that from non GM crops during seed production, the existing seed production guidelines that are used internationally to isolate and segregate cultivars are likely to apply (e.g., [39,41]). It is also worth noting the development of a range of marker tools for the determination of varietal purity based on plant [42,43] or endophyte [44] DNA are now available to assist with varietal identification and seed production QA, along with the previously mentioned methods to detect transgenes to assess not only the presence or absence of GM seeds but also the background in which these events occur. It is also possible for industry to develop protocols to further minimize gene flow between GM crops and those being grown for markets sensitive to GM such as the "Grower Opportunity Zones" or GOZ as defined by the National Alfalfa and Forage Alliance in the USA [9].

4.2. Transport of Seed and Hay Crops

Another aspect to consider is the spillage of seed during the transport of seed and hay crops. A recent study in the US has shown that in a survey of 4190 sites on roadside verges in 2011/2012, 185 contained feral alfalfa (lucerne) populations of which 38 tested positive for the presence of the *CP4 EPSPS* transgene [45]. These authors concluded that the distribution of feral alfalfa populations was not random and tended to be clustered in seed and hay production areas where transport of seed was likely, and that efforts to minimise seed spillage during transit and eradicating feral alfalfa along roadsides would be effective strategies to minimize the flow of transgenes. They also used spatial analysis to suggest that these feral populations started independently to provide further evidence that these populations were the likely result of seed spillage or some other mechanism of seed transfer.

4.3. Isolation, Separation, and Segregation of Forage Crops

In order for gene flow to occur not only must pollen find a synchronously flowering plant, pollination must occur, a fertile and mature seed must form, and this seed must join the seed bank, germinate, and establish itself in an established pasture.

4.3.1. Seedbank and Recruitment of Perennial Ryegrass into Existing Pastures

Perennial ryegrass seeds are not persistent in the soil, forming a transient type 1 seed bank [46] due to low seed dormancy and the ability to germinate across a range of environmental conditions. For instance, only 14% of the perennial ryegrass seed bank remained 14 months after release and all had gone after 2 years [47]. Little is known about the seedling recruitment of perennial ryegrass

into established dairy pastures in Australia. However, a recent study in New Zealand showed no germination of sown grasses in contrast to weedy annual and perennial grasses [48].

Seedling recruitment of sown perennial grasses into established pastures in Australia is generally poor [49] and is considered to be a cause of the poor persistence of these pastures under grazing. However, under conditions where grazing management encourages the development of mature heads it is possible to see seedling recruitment of perennial ryegrass, particularly in marginal conditions (e.g., [50–52]). There are attempts to manage perennial ryegrass pastures to facilitate seedling recruitment in meat production systems—for instance, the following from an EverGraze guide to encourage seedling recruitment of perennial ryegrass under grazing in Australia,

- Allow pasture to increase to 3000 kg/ha by the end of November
- Remove stock from mid-November to mid-January
- Graze the dry standing feed down to 1000 kg/ha before the autumn break

It can be seen that this management is not consistent with modern management of dairy pastures, where either grazing management or fodder conservation would be used in November to handle this Spring flush rather than allowing it to go to head and have the seed ripen over a 2 month period.

4.3.2. Management Practices That Could Be Used to Further Minimise Any Gene Flow between Adjacent Forage Crops

A review of co-existence strategies for maize grain crops in the EU found that a reliance merely on isolation distances often led to legislation of isolation distances that were not based on scientific principles [6], and that management and biological issues such as

- pollen barriers
- flowering coincidence
- crop rotation
- regional strategies
- biological confinement

should all be considered when developing co-existence frameworks.

Along with these general guidelines a number of specific interventions have been proposed to facilitate the co-existence of GM and non-GM alfalfa hay crops [9,10]. These are also applicable to perennial forages grown for dairy grazing and include,

- Selecting seed that is certified for purity and quality
- Preventing transfer during harvest through cleaning machinery
- Testing to confirm non-GM status, if required

The following section of this review will address how these issues may be considered for a grazed dairy system.

Given the paucity of data on seedling recruitment in dairy pastures, it is not possible to state that the following interventions would reduce the amount of gene flow from X to Y, nor is it possible to state whether the isolation distances used in seed production could be reduced by Z. Therefore the following section describes some general principles and practices that could be used to reduce gene flow but does not seek to quantify their relative efficiency. It is also important to note that most gene flow occurs from plants that are near to each other so if the large pollen source is a paddock adjacent to a well-managed dairy pasture and this paddock is laxly grazed and allowed to set seed, then most of the pollination will occur from plants within that paddock rather than by pollen from the well managed neighbour. Regardless of the absolute amount, the relative amount of pollen shed by a paddock or plot is also based on the amount of "edge" of that paddock relative to its overall size.

So within a large square paddock most of the pollen that is shed falls within the paddock boundary (this is why seed production isolation distances do not increase as paddock size increases; in fact, the opposite is true [39]).

4.3.3. Management of the "Donor Paddock" and "Recipient Paddock"

4.3.3.1. Sow One Large Paddock as Opposed to Multiple Small Ones

This minimises the proportional amount of area for pollen shed and also in isolation areas if these are used.

4.3.3.2. Utilise Management to Avoid Flowering and Seed Set

This is consistent with modern dairy pasture management and includes both grazing and the option for fodder conservation and silage, in order to remove flower heads before anthesis and/or seed set.

4.3.3.3. Consider the Use of a Boundary Crop Sown to a Non-GM Cultivar Around the "Donor" Paddock or Farm

This area can be managed in exactly the same way as the GM pasture but its physical presence will minimise the potential for gene flow.

4.3.3.4. Consider the Use of "Reproductive" Barriers to Gene Flow Such as Flowering Time and Ploidy

Modern perennial ryegrass cultivars exist as either diploids or induced tetraploids and these two classes are effectively reproductively isolated from each other outside of the laboratory. This reproductive isolation was actually used to allow the gene flow work of Cunliffe et al. [37] to occur in a region with endemic presence of diploid ryegrass pasture. Examples of tetraploid cultivars are Bealey and Banquet, and diploid cultivars include Tolosa, AberDart, and Avalon.

There is also a wide range of flowering times in perennial ryegrass used commercially today. For instance, in Australia there is approximately a 50 day range in flowering date from early maturing types such as Barberia through to late heading types such as Shogun. However, there is a range even within maturity types. A full list of categories of all cultivars may be found in publications such as the Australian Seed Federation Pasture database [53].

Obviously the most extreme reproductive isolation would come from sowing an early maturing diploid adjacent to a late maturing tetraploid, but increased isolation (and hence reduction in gene flow) would also occur with less extreme contrasts.

4.3.3.5. Consider the Use of Shelter Belts between Farm Boundaries

As well as the physical effect of increasing the distance between neighbouring pastures, shelter belts also decrease wind flow [54] and are therefore likely to decrease gene flow.

5. Conclusions

This paper discusses issues related to the design and implementation of a framework for agricultural co-existence of GM and non-GM perennial pastures with a particular emphasis on high intensity commercial grazing systems such as dairy where it is likely that these perennial pastures will be sown. Therefore, it focuses on issues related to approved transgenic events for which there will already have been an assessment of the likely environmental impact of the GM product. For instance, in Australia this falls under the responsibility of the Office of the Gene Technology Regulator (OGTR) where the likely impact of the combination of the transgenic event and the recipient species to human health and the environment are assessed prior to approval to release.

As with the cross-pollinated grain crops, maize and canola, it would be possible to develop a co-existence framework for seed production in forage plants using existing principles that are

used for conventional forage seed production. There is likely to be less gene flow between adjacent grazed and established pastures under intensive grazing than between neighbouring grain or seed production paddocks. There are also a range of management interventions (on top of distance between neighbours) to further reduce gene flow. Therefore, it is concluded that it would be possible for industry to develop a co-existence framework for GM perennial pastures including perennial ryegrass for both seed production and grazing.

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