Effect of Plant Growth Regulators and Biostimulants with and Without Foliar Nutrition on Reduced-Lignin Alfalfa (HarvXtra®)

By

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Abstract

Known as the “Queen of Forages,” alfalfa *(Medicago sativa* L.*)* is regarded for its high protein content and palatability, making it the preferred feedstuff for high-producing dairy cows. The introduction in (2005) of transgenic, reduced-lignin alfalfa as feed to dairy cows has been found to increase milk, a result of reduced lignin and increased digestibility. Simultaneously, the use of plant growth regulators and biostimulants in alfalfa production has become a topic of interest. However, there has been very limited research conducted on the yield and quality effects of plant growth regulators and biostimulants on reduced-lignin alfalfa. The objective of this experiment was to determine effects of application of indolebutyric acid (IBA), gibberellic acid (GA), kinetin, or seaweed extracts, with and without sulfur and boron, on regrowth and quality of established reduced-lignin alfalfa. Fifteen treatments with five replications and a minimum of 2-cuttings per field were applied, harvested, and analyzed for yield and quality across three fields of reduced-lignin alfalfa in Wisconsin, Illinois, and Missouri. Quality measurements included crude protein (CP), acid detergent fiber (ADF), acid detergent lignin (ADL), neutral detergent fiber (NDF), neutral detergent fiber digestibility (NDFD), relative feed quality (RFQ), and total digestible nutrients (TDN). None of the applied plant growth regulators or biostimulants consistently increased or decreased yield or quality of alfalfa with the reduced lignin trait. Overall, there were no effects of applied growth regulators on transgenic alfalfa yield or quality. At one location, the addition of sulfur and boron sometimes increased alfalfa yield, but never quality.

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List of Abbreviations

ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
RFQ	Relative Forage Quality
NDF	Neutral Detergent Fiber
NDFD	Neutral Detergent Fiber Digestibility
TDN	Total Digestible Nutrients
CP	Crude Protein
RFV	Relative Feed Value
COMT	Caffeic acid 3-O–methyltransferase
CCOMT	Caffeoyl CoA 3-O-methyl- transferase
PGR	Plant Growth Regulator
ivTDM	Dry Matter
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
GA	Gibberellic Acid
CAD	Cinnamyl Alcohol Dehydrogenase
FGI	Forage Genetics International
N	Nitrogen
S	Sulfur
B	Boron
NIR	Near-Infrared

1. Literature Review

1.1 Alfalfa and the United States

Modern alfalfa (*Medicago sativa L. subsp. Sativa;* also known as 'lucerne') is a genetically synthetic cultivated type autotetraploid (2n=32) forage crop native to Turkey with approximately 800 to 900 mega base pairs (Kumar, 2011). Geneticists believe alfalfa to be derived from M*edicago caerulea*, L. (2n=16), but research has not yet confirmed this idea (Havananada et al., 2011). Alfalfa was first introduced into the United States during the colonial era, but repeatedly failed to successfully establish. A second, successful attempt was made around 1854, when alfalfa was brought north from Chile to San Francisco, beginning the widespread production on irrigated land. Several years later, alfalfa establishment was successful on non-irrigated land, further expanding the opportunity to grow alfalfa as forage for dairies across the United States (Westgate, 1908).

In 2015, United States alfalfa production occurred on 7.2 million hectares, with the top six states (SD, MT, ND, WI, MN, ID) contributing 47% of the total harvested hectares (Scuse and Parsons, 2016). Growers produced 53.5M metric tons of alfalfa, with California in the lead at 4.9M metric tons, followed by Idaho (3.8M metric tons) and South Dakota (3.8M metric tons). However, when yield is considered on a per hectare basis, Arizona leads in the US with an average of 18.8 metric tons ha^{-1}, followed by California (15.5 metric tons ha^{-1}) and Washington [11.7 metric tons ha^{-1}; Johansson and Harris, 2016]. In California, dairy cows consume 75% of the alfalfa harvested in the state, which account for 21% of the dairy products produced in the entire U.S. (Putnam et al., 2007).

While alfalfa is grown around the world, the United States is the leader in forage dry matter production and research for improving production characteristics such as yield, quality, and herbicide resistance. South American alfalfa production is second but only makes up about 23% of the total forage dry matter yield produced in the United States (Food and Agriculture Organization of the United Nations, 2016). In ranking order, Japan, China, Korea, the United Arab Emirates, and Taiwan are major importers of U.S. alfalfa for use in livestock systems, as each country's dairy industry is growing.

Alfalfa grown in the US is predominantly exported from ports along the western seaboard, but only consists of 3.5% of all U.S. alfalfa production (Putnam et al., 2015). Alfalfa production in the US is typically reported as tons of dry matter rather than metric tons, and the price of alfalfa has a broad range depending on quality, the local market, and scale of forage purchased (i.e. individual bale package basis or multiple lots). For instance, in California's Northern San Joaquin Valley, alfalfa graded as "fair" sold for $110/metric ton, while a "supreme" grading sold for $269/metric ton. However, in Southern California, fair-graded alfalfa will not sell at $110/metric ton, and the top end price is much lower than in the Northern San Joaquin Valley, at $205/metric ton. Thus, depending on where alfalfa is sold, the low- and high-end prices vary. The picture looks similar across the country, where price is determined based on systems use at the local level (USDA, 2016).

Alfalfa is highly regarded for its high protein concentration, digestibility and palatability (Putnam et al., 2007). The main use of alfalfa is as forage or a feed supplement for animals in the livestock (dairy, beef, small ruminants) and lifestyle (horses, farm pets) industries, with dairy being the primary consumer (Putnam et al., 2001). It is also utilized to improve soil health, serve as a wildlife habitat and insectary, and mitigate contamination from industrial, dairy, and municipal wastes. Livestock performance, especially in dairy systems, and forage quality are primary drivers of alfalfa/forage quality research. The dairy industry has set the standard in forage quality for many years, and as a result, the rest of the agriculture industry has benefitted from the high-quality feed alternative to grass silage or hay (Chen et al., 2006).

Although it is a legume, alfalfa is less frequently used as a cover crop for other crops of economic importance (Chen et al., 2006). This is partly because the seed is expensive, and the crop is thus far more likely to be managed as a perennial rather than an annual cover crop. However, since alfalfa is a legume it will fix N, and residual N is available to subsequent crops (Yost et al., 2014). Estimates of fixed N available for the succeeding crop will vary but are typically in the range of 40 to 200 kg ha^{-1} yr^{-1} (Mohr et al., 1999).

1.2 Forage Quality and Measurements Used for Description

Forage quality is defined and measured under several parameters, with the simplest definition being that it is a quantitative correlation to the potential efficiency of livestock production (i.e. milk, beef, wool) through available nutrient use from forage crops (Barnes and Marten, 1979). Common mechanisms of measurement for forage quality include animal performance measures and/or chemical composition analyses including acid detergent fiber (ADF), relative forage quality (RFQ), neutral detergent fiber (NDF), neutral detergent fiber digestibility (NDFD), total digestible nutrients (TDN), and crude protein (CP).

Relative forage quality (RFQ) is an improved calculation over RFV (relative feed value) as RFQ considers total digestible nutrients and fiber digestibility, providing a good indication of forage quality (Jeranyama and Garcia, 2004). Crude protein (CP) is derived through calculation, utilizing the Kjeldahl method for determining nitrogen, and indicates the capability of a feed to meet the livestock's protein needs (Sáez-Plaza et al., 2013; Bremner and Breitenbeck, 1983). An ideal RFQ value of alfalfa for milking dairy cows is 140 to 160, while the typical CP value ranges from 18 to 25% on a dry matter basis. While each of the above measurements serves a unique role in forage quality analysis, the key parameters of interest for this review include ADF and NDF, as they quantitatively define lignin in a feedstuff. Typical values for ADF and NDF in quality alfalfa are less than 35% and less than 45%, respectively (Hancock and Collins, 2006).

Neutral detergent fiber (NDF) is the measure of the lignin, cellulose, hemicellulose, and ash percentage that is digested in a specific time frame from feed/forage. Neutral detergent fiber does not include pectin as pectin solubilizes and cannot be measured with the neutral detergent technique. Neutral Detergent Fiber Digestibility (NDFD) is the measure of digestibility of the NDF over a specific time frame. This time frame is typically measured over 24, 30, or 48 hours. The higher the NDFD of a feedstuff, the lower the amount of energy an animal can obtain from it (Undersander et al., 2004; Jung, 2004). Twenty-four-hour NDFD value ranges for alfalfa hay are 27% (low), 32% (medium) and 39% (high). Thirty-hour NDFD value ranges are 33% (low), 40% (medium), and 49% (high). Forty-eight-hour value ranges for alfalfa hay are 44% (low), 50% (medium), and 55% (high) (Cassida et al, 2000), and are often the values used in the calculation of RFQ.

Acid detergent fiber is defined as the measure of the percentage of slowly digestible and highly indigestible feed/forage material, including cell wall components such as lignin, cellulose, and ash (Undersander et al., 2004). Acid detergent fiber is extracted following the measure of NDF by boiling the lignin, cellulose, hemicellulose, and ash in acid detergent solution (Goering and Van Soest, 1970). Although acid detergent methods have been found to be inaccurate with low measure of true lignin in forages, acid detergent lignin (ADL) is the primary method utilized by laboratories to determine lignin concentration in feed (Jung et al., 1997). Acid detergent lignin is isolated from ADF via a 72% sulfuric acid extraction (Van Soest, 1963).

During alfalfa maturation, lignin, a key component in the structural integrity of cell walls, increases, while protein content decreases, reducing the overall digestibility of the feedstuff, as lignin is indigestible to ruminant animals (DePeters, 2013; Undersander et al., 2009). Lignin's role in the plant is to provide mechanical strength via cross-links with cellulose and hemicellulose in the secondary cell wall, and to provide hydrophobicity, preventing water leakage out of the vascular tissue (Undersander et al, 2009; Lewis and Yamamoto, 1990).

1.3 Considerations for High Quality Alfalfa Production

1.3.1 Time of Cutting

A key consideration for dairy producers managing alfalfa is harvest interval and stage of growth at harvest, as forage quality declines with increasing plant maturity (DePeters, 2013). In Wisconsin, growers with dairy cows fed high quality alfalfa reported a $400 per cow profit in milk production over cows fed low-quality alfalfa. These results are further supported by data that showed alfalfa had 16 to 20% crude protein when harvested early in the season at the late-bud to early-bloom stage and 12 to 15% for late-season harvest when compared to grass hay, which averaged 8.4% crude protein on a dry matter basis. The same results were found for fiber quality, with average fiber content of alfalfa of 24%, while grass hay averaged 31.4% fiber (Balliette and Torell, 1993). The lower the fiber content, the higher the digestibility. So, since grass contains greater levels of fiber, it can take cows nearly twice as long to digest. This slows the rate of milk production in dairy cows and rate of weight gain in beef cattle (Balliette and Torell, 1993). Thus, producers aiming for high, efficient output from livestock aspire for feed to contain moderate-to-high protein content and low fiber.

1.3.2 Reduced Lignin

While high lignin content is an extremely valuable characteristic for many field crops (e.g. corn), the opposite is required for high-quality feed and forage, as high lignin concentration holds an inverse relationship with forage quality standards for digestibility (i.e. NDFD) (Smith et al., 1972). Researchers have focused on reducing lignin content in alfalfa without tarnishing all plant structural integrity, as lignin is a source of fiber, and reducing fiber results in better digestibility, higher forage quality, and thus, a more productive dairy cow.

1.3.3 Technological Advancements in Alfalfa Genetics

There are three primary genetic suppliers of commercially available alfalfa: Forage Genetics International, Alforex Seeds, and S & W Seed Company (NAFA, 2016). These are commercial companies which all develop their own traits and varieties. Conventional commercial alfalfa propagation is mostly conducted through cross-pollination of two nursery-grown parent types. The F_1 generation goes to seed production and is tested in yield trials for multiple years until top hybrids are identified (Forage Genetics, 2016). Utilization of biotechnology in breeding opened up the possibilities for improving alfalfa hybrid (intraspecific) yields with the 2005 initial launch of glyphosate-tolerant alfalfa (Gaston, 2005).

Since 2005, Forage Genetics International (N5292 Gills Coulee Rd. S, West Salem, WI 54669), in conjunction with the University of Wisconsin and several other contributors, found an approach to reduce lignin through down-regulation of the caffeic acid 3-O–methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCOMT) lignin biosynthesis genes in transgenic—Roundup Ready® alfalfa. As a result, reduced-lignin alfalfa cultivars were released to the market under the brand name HarvXtra® (Undersander et al., 2009). A conventionally bred line, meaning no transgenic gene down-regulation was included, was released around the same time by Dow's Alforex Seeds (N4505 Co Hwy M, West Salem, WI 54669) under the brand name Hi-Gest®.

In the transgenic lines, the COMT down-regulated gene showed a reduction in S/G lignin ratios with a near total reduction in the S-lignin compound, and a reduction in G-lignin. The CCOMT down-regulated gene provided a reduction in G-lignin, and no reduction in S-lignin *in vitro* (Guo et al., 2001). Reduced-lignin alfalfa lines, along with their null isogenic lines and a variety check, were grown in diverse climates to determine how yield and quality of the transgenic lines performed versus controls. Results indicated that transgenic alfalfa harvested on a 3-cutting harvest schedule in the same time frame of a 4-cutting harvest schedule yielded 20-30% higher than non-transgenic alfalfa, suggesting a producer had the option to delay cutting by 8 to 12 days to maintain forage quality of a 4-cutting schedule, or they could cut higher quality alfalfa on the same 4-cutting schedule, as compared to non-transgenic lines. As alfalfa regrows after a cutting, its rate of growth is slow. However, as the crop matures, the rate of growth increases. Thus, in reduced-lignin alfalfa where quality is not compromised by delaying harvest, yield can dramatically increase compared to non-transgenic lines where quality is compromised on a 3-cutting schedule. Transgenic COMT and CCOMT lines decreased ADL by 3.7% and 12%, as well as increased NDFD 14% and 10.2%, respectively (Undersander et al., 2009). Either decision has potential to increase a producer's profitability without sacrificing current forage quality standards on the farm.

1.4 Plant Performance Factors

1.4.1 Plant Hormones and Plant Growth Regulators

Plant hormones are naturally occurring, organic substances that play crucial roles in plant growth and development. Examples of plant hormones include auxins, gibberellins, cytokinins, abscisic acid, ethylene, jasmonates, brassinosteroids, salicylic acid and polyamines (Thomas and Thomas, 1979). A plant growth regulator (PGR) is classified as a chemical utilized to accelerate or inhibit a plant's root, shoot, and sucker growth, delaying of harvest, promotion of fruit and seed development, plant strength, blossom set, or fruit size. The compound must induce a plant response beyond that of standard nutrition (Davies, 2010).

Key plant growth hormones commonly discussed in the agriculture industry today can be separated into three groups: 1. Growth promoting—auxins, gibberellins, cytokinins, brassinosteroids, 2. Senescence promoting/growth inhibition—abscisic acid, ethylene, jasmonates, and 3. Plant defense—abscisic acid, salicylic acid, and jasmonates (Häffner et al., 2015). This thesis focuses on growth promoting PGRs, as the goal of an alfalfa grower is to increase dry matter (ivTDM) without sacrificing forage quality.

1.4.2 Auxin

Auxins are known to function at the junction between environment/development and the pathways of response that they trigger. Indole acetic acid (IAA) is the homeostatic form of auxin found in plants and can be produced via several precursors to solicit a growth response. *De novo* synthesis of IAA begins with tryptophan, as it contains all of the necessary carbon and structures to generate IAA. An example of an endogenous and exogenous IAA precursor is indole butyric acid (IBA), which is found in commercial seedling growth-promoting products. The role auxins serve in the plant is plentiful starting with stimulation of cell division and enlargement, vascular tissue differentiation, root initiation, as well as the well-studied tropic responses and apical dominance. Auxins also function to delay leaf senescence, abscission, and fruit ripening (Cheng et al., 2007). There is limited published research which examines the use of auxins in alfalfa production (Cowett and Sprague, 1962).

1.4.3 Gibberellic Acid

Gibberellic acid (GA) encompasses numerous forms. In fact, there are over 125 versions of the *ent*-gibberellane structures known today. Many of the GA products available commercially are in the GA_3 isomer form, but there are a few other isomers found in proprietary commercial products. GA_n forms convert to GA_1 or GA_4 once taken up by a plant in order to take a bioactive form. Once the hormone is converted to an active form, it plays a crucial role in enzyme production during seed germination, and stem growth, especially in response to light and long days (Davies, 2010). Limited work has examined the use of gibberellin acid for alfalfa. One study that included gibberellic acid as a seed treatment in alfalfa (of five PGRs studied) found that seed yield was increased whenever a PGR was applied, without affecting seed quality (Zhang et al., 2009).

1.4.4 Cytokinin: Kinetin

Kinetin was the first cytokinin discovered that is not naturally occurring in plants. Though synthetic, kinetin plays a major role in influencing cell division, morphogenesis, lateral bud growth, shoot initiation and elongation, and leaf senescence. Cytokinins also play a role in chloroplast development and stomatal opening (Farber et al., 2016).

While there has been extensive research on PGRs in other field crops, little research has been conducted regarding the effects of PGRs on alfalfa yield and quality

(Massengale and Medler, 1958). In one study conducted in 1991, cytokinins (kinetin and BAP) were applied foliarly to low- and high-density alfalfa stands immediately after harvest and 3 to 7 days after harvest. In all scenarios, alfalfa dry matter increased, while forage quality remained unaffected in the field and reduced in the greenhouse (decrease in CP and increase in ADF, a result of increased stem diameter). The increase in dry matter overshadowed the reduction in forage quality (Tompkins and Hall, 1991). When the auxin to cytokinin ratio was high, alfalfa had a reduction in axillary buds, causing fewer shoots per plant (Cowett and Sprague, 1962). This limits the potential for a denser stand, and potentially higher ivTDM yield.

1.5 Lignin Biosynthesis and Sneaky PGRs

While the down-regulation of COMT and CCOMT genes is considered to be a break-through innovation in alfalfa research, it potentially left a gap in the lignin biosynthesis pathway that could allow for biosynthesis to still occur under at least two scenarios (See Fig. 1 for Lignin Biosynthesis Pathway adapted from Cesarino et al., 2012). The pathway starts with the essential amino acid, phenylalanine, and through several conversions of the macromolecule, three monolignols are produced with the help of the enzyme cinnamyl alcohol dehydrogenase (CAD). They are then oxidized by peroxidases and/or laccases to form S-lignin, G-lignin, and H-lignin (Cesarino et al., 2012). The down-regulation of COMT and CCOMT prevent the production of S-lignin and G-lignin, but leaves an available pathway for H-lignin to be produced—scenario one. The second scenario where lignin biosynthesis could bypass the down-regulation of COMT and CCOMT would be if there were endogenous or exogenous influxes of plant hormones or plant growth regulators, respectively, stimulating the production of CAD, thus allowing feedback to stimulate lignin production.

There are at least twelve known genes that play a role in lignin biosynthesis, but there is suggestion that the monolignol pathway is over-simplified, and there are more genes affecting lignin biosynthesis in alfalfa (Chen et al., 2006). Auxins, specifically endogenous IAA, are known to increase CAD (cinnamyl alcohol dehydrogenase) expression (Kim et al., 2007), which is a vulnerable step between the down-regulated COMT gene in transgenic reduced-lignin alfalfa and prevented production of S-lignin in vascular tissue, as the expression of CAD is down-stream from COMT gene expression and is well-documented as being the last key step in the biosynthesis of S-lignin. Exogenous IBA converts to IAA once in the plant. Thus, applications of IBA could result in an increase in lignin biosynthesis. However, the effects of an application consisting of IBA, kinetin, and GA are not understood. The objective of this experiment was to determine effects of application of indolebutyric acid (IBA), gibberellic acid (GA), kinetin, or seaweed extracts, with and without sulfur and boron, on regrowth yield and quality of established reduced-lignin alfalfa.

2. Materials and Methods

2.1 Background

Experiments were conducted for 1 year in 2018 at each of 3 locations: 1) Forage Genetics International (FGI) (43° 54' 33.732'' N, 91° 7' 15.564'' W) in West Salem, Wisconsin; 2) Graybill Farms (42° 21' 30.996'' N, 89° 32' 16.692'' W) in Dakota, Illinois; and 3) Purina Animal Nutrition (38° 29' 58.632'' N, 90° 49' 2.352'' W) in Gray Summit, Missouri. Soil types and basic background soil information can be found in Table 1. Plot size was 1 x 1.5 meters (FGI) and 1.5 x 3 meters (Purina Animal Nutrition and Graybill Farms) with treatments arranged in a randomized complete block design with five replications of each treatment. Supplemental irrigation was not provided, and plots were not inoculated prior to seeding. Soil nutrients applied at seeding were unknown. The fields were treated with 420 ml ha^{-1} pyraclostrobin fungicide (Headline® Group 11 strobilurin; BASF 26 Davis Drive, Research Triangle Park, NC 27709) prior to the first cutting of the season to safeguard against disease. Cultivars at each of the plots were HarvXtra® HarvaTron (Forage Genetics International: N5292 Gills Coulee Rd. S, West Salem, WI 54669).

West Salem (FGI) plots were seeded at 13.5 kg ha^{-1} on May 12, 2017 with a Carter walk-behind seeder, were flooded out in June, and re-seeded at the same rate on July 3, 2017. Alleyways of 0.31 meters separated each plot from east to west and 0.91-meter alleys from north to south. Treatments (Table 2) were applied in 2018 to the second-year stand alfalfa, with initial treatments applied on May 24, 2018, the second treatments applied on June 28, 2018, and final treatments applied on July 15, 2018.

The Purina Animal Nutrition location was seeded at 16.8 kg ha^{-1} in May 2016 and the experiment was conducted on the third-year crop, with initial treatments applied on May 29, 2018 and final treatments applied on June 20, 2018.

Graybill Farms was seeded at 13.5kg ha^{-1} in May 2017 and the experiment was conducted on the second-year crop, with initial treatments applied on June 7, 2018 and final treatments applied on July 12, 2018.

2.2 Applications

The materials and methods used for application were the same for each location. Each round of applications were made when alfalfa regrowth was 15-20 cm after the prior harvest—approximately eight days post-harvest. All applications were tank mixed with polyoxyethylene sorbitan fatty acid ester adjuvant to mitigate drift (InterLock®; WinField Solutions, LLC P.O. Box 64589, St. Paul, MN 55164) and sprayed 0.5 m above the canopy with an R&D Sprayers® (419 LA-104, Opelousas, LA 70570) CO_2-operated backpack sprayer and boom using TeeJet® AIXR 11002 nozzles at 45 psi to maximize coverage and minimize drift at a 140 liters ha^{-1} rate.

There were fifteen treatments (Table 2) that consisted of various combinations of plant growth regulators, biostimulants, sulfur (S), and boron (B). The plant growth regulator ingredients consisted of three growth-promotors in various proprietary combinations: indolebutyric acid, gibberellic acid, and cytokinins as kinetin. The biostimulants tested were classified as protein hydrolysates or seaweed extracts with various ingredients including the amino acids tryptophan, arginine, fenugreek, wheat germ or the cold-extracted compounds from *Ascophyllum nodosum,* respectively.

All treatments also contained Lambda-cyhalothrin at 134ml ha^{-1} (Grizzly® Too insecticide; Group 3 pyrethroid; WinField Solutions, LLC P.O. Box 64589, St. Paul, MN 55164) to remove insect pressure as a variable.

2.3 Tissue Samples

Tissue samples were pulled as a sub-sample immediately following hand harvest on two replications and two cuttings at Purina Animal Nutrition to determine S and B concentrations in each treatment. Samples were taken from the top 25-30 cm of the crop and were Next-Day air mailed to SureTech Laboratories in Indianapolis, IN for nutrient analysis. SureTech Laboratories conducts closed-vessel digestion for minerals and analyzes nutrient concentrations on an Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) (ThermoFisher Scientific; 81 Wyman Street, Waltham, MA 02451) and the automated combustion method using a thermal conductivity detector for nitrogen analysis (Elementar; 119 Comac Street, Ronkonkoma, NY 11779) (Kalra, 1998).

2.4 Harvest and Data Collection

Harvest was maintained on a 28-day schedule for consistency, as is typical across the United States. Each plot was treated and harvested twice for data with the exception of West Salem, which was treated and harvested three times.

The West Salem location was harvested with a Carter Flail Forage research harvester, cutting the entire 1 x 1.5 m plot. Purina Animal Nutrition and Graybill farms were cut with hand pruners using a 0.28 m^2 square. The square was placed in the center of the treated replications on the ground, and harvest was achieved by cutting all foliage

1-inch above the tops of the crowns falling within the parameter of the square. Samples were bagged in labeled, breathable tissue-sampling bags.

Sample were placed either in the greenhouse in open trays (West Salem and Graybill Farms) or in the bag (Purina Farms) in a forced air laboratory drying oven set to 46^{oC} for 48 hours before dry weights (yield) were recorded with a gram scale. All samples were analyzed for quality at Forage Genetics International. Samples were ground with a Wiley mill to a 6mm grind, followed by a fine grind of 1mm with a Udy cyclone mill. Alfalfa quality was analyzed via NIR spectroscopy using a FOSS NIRS DS2500.

2.5 Statistical Analysis

Repeated measures analysis was used to determine whether treatment differences were observed in alfalfa yields and quality. Two models were used. The first model went across all locations and location was considered a random factor. The second model analyzed the data by individual location and all factors were considered fixed. Proc Glimmix was used in SAS 9.4 (SAS Institutes, Inc. Cary, NC, USA) with alpha =0.1. Mean separation was performed using Tukey's adjustment. Linear contrasts were used to determine differences between control and all other PGR x Fertility interaction, control vs. any PGR alone, PGR alone vs. PGR + Fertility, Fertility alone vs. PGR alone, Fertility alone vs. PGR + Fertility, Ascend SL vs. Ascend SL + Fertility, Toggle vs. Toggle + Fertility, and Ascend + Toggle vs. Ascend + Toggle + Fertility.

3. Results and Discussion

Initial analysis of variance for all measured data variables indicated that the location by treatment interaction was never significant (Table 3). However, the main effect of location was always significant. Thus, for much of this thesis results will be discussed by location.

3.1 Alfalfa Yield by Cutting and Location

3.1.1 Illinois Location

Alfalfa yield was unaffected by treatment in either the June or July cutting date (Tables 4 and 5). None of the applied materials ever significantly increased or decreased yield of alfalfa, as compared to that measured in the untreated plots. Overall, yields were high and comparable to average alfalfa yields for the region, which typically are between 4.48 and 11.2 Mg ha^{-1} (USDA, 2018).

At no time did the application of supplemental nutrients (sulfur or boron) affect yield. Others have evaluated these nutrients for alfalfa, and also found no yield to addition of boron (Sapkota et al., 2019). In that work, boron was added up to 2.24 kg B ha^{-1}, and although petiole boron increased yield did not. In other work foliar B (up to 1200 mg B L^{-1}) did improve seed set and seed yield, but effects on forage yield were not measured (Dordas, 2006).

For sulfur, most work was done long ago (1960s) and in sulfur-deficient soils. In these cases, application of sulfur did increase alfalfa yield (Seim et al., 1969; Pumphrey and Moore, 1965; Caldwell et al., 1969). That work was all performed on sulfur-deficient soils, which was not the case in our studies. Unfortunately, initial soil tests were not collected for this work, and so we cannot directly discuss soil-test sulfur. However, basic soil types (Table 1) would lead us to believe sulfur deficiencies were not likely, and the lack of response to applied sulfur is not unexpected.

3.1.2. Purina Farms Location

In June, neither yield nor quality variables were affected by any treatment (Table 6). However, in the July cutting yield was affected by treatment, as were some quality analyses (to be discussed later) (Table 7).

At Purina Farms, alfalfa treated with A*scophyllum nodosum* (Toggle®), sulfur, and boron (Treatment 12) yielded higher than that of the control, or from the alfalfa from treatments 3, 6, 8, 9, 10, 13, 14, which included various combinations of IBA, kinetin, and GA, and the alfalfa from treatment 14, which contained GA, sulfur, and boron (Table 8). However, the alfalfa from treatment 12 yielded statistically the same as the alfalfa treated with sulfur and boron only, protein hydrolysate only, gibberellic acid only, a combination of protein hydrolysate, sulfur, and boron, and when the alfalfa was treated with IBA, kinetin, GA, *Ascophyllum nodosum*, sulfur, and boron.

So, when sorted for all the various treatments the 3-way mix of Toggle (sea kelp), sulfur and boron increased yield as compared to the control, but not when compared to those materials applied alone. For example, application of Toggle alone (Treatment 4) did not improve yield when compared to the control. Similarly, application of sulfur and boron alone had no beneficial effects on yield (Table 8).

Between the application date (June 20, 2018) and cutting date (July 17, 2018) at Purina Farms, the average daily temperature was 28.3°C and the average daily temperature high was 32.2°C. During those 28 days, the Purina Animal Nutrition area received 89 mm of precipitation (wunderground.com, 2021). The high average daily temperature in conjunction with low rainfall suggests the alfalfa at Purina Farms was under stressed conditions during the regrowth period following the June 20, 2018 treatments, and thus that may have been why some differences were observed in the July cutting.

Ascophyllym nodosum extract products have been found to reduce the stomatal conductance in soybean plants under heat and water stress (Martynenko et al., 2016). Sulfur has shown to aid with abiotic stress (Saito, 2000). Boron has shown to enhance gene expression that regulate antioxidant enzyme activity (Aydin et al., 2019). This suggests that the *Ascophyllum nodosum* extract, sulfur, and boron in Treatment 12 may have aided the alfalfa in overcoming heat and drought stress better than the control or any of the products applied from treatments 3, 6, 8, 9, 10, 13, and 14 which contained IBA, kinetin, and gibberellic acid, resulting in a higher yield. Though IBA, kinetin, and gibberellic acid are all known to promote growth, the ratio of such compounds in a plant can cause various effects as cited in the literature review of this paper. In this study, an objective was to determine which products may increase yield without negatively affecting digestibility. The alfalfa treated with *Ascophyllum nodosum*, sulfur, and boron fulfilled that objective, but only as a three-way combination. Additionally, it should be noted that this was only observed in one cutting at one location (Table 8). And such an effect was not observed when cumulative yields were analyzed (Table 9).

Also important to note, application of treatments 2, 4, 5, 7, 11, and 15 produced alfalfa with the same yield as that measured in alfalfa to which treatment 12 (*Ascophyllum nodosum,* sulfur, and boron) was applied. Treatment 2 (sulfur and boron), Treatment 4 (*Ascophyllum nodosum*) were the individual combinations that made up Treatment 12, suggesting further study should be considered in order to fully determine if the collective application of *Ascophyllum nodosum*, sulfur, and boron is necessary to aid in drought tolerance or if the individual component applications could suffice. Alfalfa to which Treatment 5 (protein hydrolysate) and Treatment 11 were applied had the same yield, with the only difference in the treatments being the addition of sulfur and boron in Treatment 11, suggesting the addition of sulfur and boron did not increase alfalfa yield. Again, further examination may be necessary to understand the nuances across various environments, as there was no statistical difference in cumulative yield at any of the three locations (Table 9).

3.1.3 West Salem Location

Alfalfa yield was unaffected by treatment in the two early cuttings (June and July (Tables 10 and 11), but yield was affected at the last cutting (August, Table 12). In this location, significant effects occurred because application of various products reduced alfalfa yield below that measured in the control plots, rather than increasing yield. Specifically, application of IBA, GA and kinetin (Treatment 9), seaweed extract, sulfur and boron (Treatment 12) and experimental rates of IBA, GA and kinetin, plus sulfur and boron (Treatment 13) reduced alfalfa yield below that measured in the control plots (Table 13).

Similar to results observed in the Purina Farms July cut (Table 8), significant differences shown by various combinations of materials were not observed when solo materials were applied. For example, application of the experimental blend of IBA, GA and kinetin alone (Treatment 6) had no effect on alfalfa yield, but when the sulfur and boron were added it decreased yield (all as compared to the control) (Table 13). Similarly, application of only seaweed extract (Treatment 4) did not affect yield, but when it was added to a sulfur and boron spray, yield was reduced (as compared to the control). However, application of only sulfur and boron (Treatment 2) had no effect on yield (Table 13).

Between the application date (July 15, 2018) and cutting date (August 3, 2018) at West Salem, the average daily temperature was 20°C (wunderground.com, 2021). The average daily temperature high was 28.3°C, while the average daily temperature low was 14.4°C (wunderground.com, 2021). During those 19 days, the West Salem area received 17 mm of precipitation (wunderground.com, 2021). In another study where alfalfa was grown in controlled environment chambers for 89 days in 16 different day/night temperature regimes, it was observed that alfalfa grew best with day/night temperatures of 22/25°C, 29/18°C, and 29/25°C (Patterson, 1993). The alfalfa tested at West Salem between July 15, 2018 and August 3, 2018 experienced similar day/night temperatures *in situ* as those conducted in the 1993 climate-controlled experiment. Thus, the alfalfa at West Salem grew in the best temperature conditions possible to support growth.

3.2 Quality Indices, by Location

3.2.1 Illinois Location

No quality indices were ever affected by treatments, at either the June (Table 4) or July (Table 5) cutting.

3.2.2 Purina Farms Location

Quality indices were unaffected by treatment at the June cutting (Table 6). However, as with yield, several quality indices were affected by treatment in the July cutting (Table 7). Those significantly affected by treatment were RFQ, ADL, CP, NDF, IVdOM, ivTDM, and TDN (Table 7).

Though there were statistical differences in the relative feed quality (RFQ) in the July cutting (Table 8), all values were above the desired range of 140-160 (Undersander, 2003), indicating all combinations would be acceptable to feed a dairy cow without negatively impacting digestibility. Because RFQ is a good indicator of overall alfalfa quality, no further examination of the other quality values was deemed necessary for this discussion. However, the results were included in Table 13 for visibility. Had there been major differences in RFQ, it would have been necessary to take a closer look at the acid detergent lignin (ADL) values for this thesis as it is the measurement that best describes the amount of lignin in a feedstuff and the measurement of interest when determining if applications of PGRs and biostimulants had an effect on reduced-lignin alfalfa. However, there was no significant change observed over the control.

3.2.3 West Salem Location

No quality indices were ever affected by treatments, at either the June (Table 10) or July (Table 11), or August (Table 12) cutting.

3.3 Quality Indices, Averaged Over Location

Since quality indices were rarely affected by treatment, results were pooled over location and cutting to see if any differences could be determined from this larger data pool. The result was that none of the quality indices were affected by treatment (Table 14).

3.4 West Salem’s Weather Conditions versus Purina Farms’ Weather Conditions

While West Salem received less rainfall than Purina Farms (71 mm less) when comparing the data where statistical differences in yield occurred at the respective sites, there was a key difference observed between the two sites that may better explain why results were not mirrored at both sites. Not only were West Salem’s average daily air temperatures lower than Purina Farms by 8.3°C, but the alfalfa at West Salem observed far lower average nighttime temperatures (8°C lower) as well. Plant growth regulators have been observed as a tool for combatting yield loss due to high nighttime temperatures in other crops (Mohammed and Tarpley, 2011), supporting the hypothesis that Purina Farms saw a response due to drought stress and that it is likely that the West Salem alfalfa was provided the time needed to recover from the day's heat during the evenings, preventing a loss in yield potential in the control (Hall, 1992; Mohammed and Tarpley, 2009). The alfalfa at West Salem may not have seen a yield effect from applications of growth promoting plant growth regulators or stress-reducing biostimulants over the control because the conditions were not conducive to elicit a response.

4. Conclusion

In this study, the use of plant growth regulators and biostimulants on reduced-lignin alfalfa did not affect quality or yield across three locations and was nuanced by location and time of cutting. Further research on the proper ratios of indolebutyric acid, gibberellic acid, and kinetin, various combinations of biostimulants with and without S and B nutrition, and the effects that idyllic alfalfa growing conditions do or do not have on PGR and biostimulant response is needed to better understand the yield and quality nuances that are created when one or more of those factors changes in a reduced-lignin alfalfa system.

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Table 1: Soil Information for each selected site, alfalfa experiment, 2018.

Field Location	**MUSYM***	**Soil Description**	**Taxonomic Class**	**Draining Class**	**Soil Texture**
FGI, West Salem	336A	Toddville silt loam, 0 to 2% slopes.	Fine-silty, mixed, superactive, mesic Typic Argiudolls	Moderately well drained	Silt loam
Graybill Farms, Illinois	86B	Osco silt loam, 5 to 10% percent slopes	Fine-silty, mixed, superactive, mesic Typic Argiudolls	Well drained	Silty clay loam
Purina Animal Nutrition, Gray Summit, Missouri	60037	Wrengart silt loam	Fine-silty, mixed, active, mesic Fragic Oxyaquic Hapludalfs	Moderately well drained	Silt loam

*MUSYM: Map Unit Symbol is a shortened string for the Map Unit Name.

Table 2: Treatments applied to alfalfa in 2018 at three locations.

Treatment Number	Active Ingredient	Brand Name	Product Class	Rate	Application Dates		
					Purina Farms	West Salem	Graybill Farms
1 (Control)	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}	05/29/2018 06/20/2018	05/24/2018 06/28/2018 07/15/2018	06/07/2019 07/12/2018
2	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
	0-0-19-13S[†‡]	Max-In® Sulfur	Foliar Micronutrient	5.6L ha^{-1}			
	0-0-0-8B[¥]	Max-In® Boron	Foliar Micronutrient	1.4L ha^{-1}			
3	Indolebutyric acid, gibberellic acid, and kinetin	Ascend® SL	Plant Growth Regulator	469ml ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
4	0-0-1	Toggle®	Seaweed Extract	2.8L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
5	5-8-4	Kriss™	Protein Hydrolysate	1.12L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
6	Indolebutyric acid, gibberellic acid, and kinetin	AGM16013	Plant Growth Regulator	469ml ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
7	Indolebutyric acid, gibberellic acid, and kinetin	AGM16011	Plant Growth Regulator	469ml ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
8	Indolebutyric acid, gibberellic acid, and kinetin	Ascend® SL	Plant Growth Regulator	469ml ha^{-1}			
	0-0-1	Toggle®	Seaweed Extract	2.8L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
9	Indolebutyric acid, gibberellic acid, and kinetin	Ascend® SL	Plant Growth Regulator	469ml ha^{-1}			
	5-8-4	Kriss™	Protein Hydrolysate	1.12L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
10	Indolebutyric acid, gibberellic acid, and kinetin	Ascend® SL	Plant Growth Regulator	469ml ha^{-1}			
	0-0-19-13S	Max-In® Sulfur	Foliar Micronutrient	5.6L ha^{-1}			
	0-0-0-8B	Max-In® Boron	Foliar Micronutrient	1.4L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
11	5-8-4	Kriss™	Protein Hydrolysate	1.12L ha^{-1}			
	0-0-19-13S	Max-In® Sulfur	Foliar Micronutrient	5.6L ha^{-1}			
	0-0-0-8B	Max-In® Boron	Foliar Micronutrient	1.4L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
12	0-0-1	Toggle®	Seaweed Extract	2.8L ha^{-1}			
	0-0-19-13S	Max-In® Sulfur	Foliar Micronutrient	5.6L ha^{-1}			
	0-0-0-8B	Max-In® Boron	Foliar Micronutrient	1.4L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
13	Indolebutyric acid, gibberellic acid, and kinetin	AGM16013	Plant Growth Regulator	469ml ha^{-1}			
	0-0-19-13S	Max-In® Sulfur	Foliar Micronutrient	5.6L ha^{-1}			
	0-0-0-8B	Max-In® Boron	Foliar Micronutrient	1.4L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
14	Indolebutyric acid, gibberellic acid, and kinetin	AGM16011	Plant Growth Regulator	469ml ha^{-1}			
	0-0-19-13S	Max-In® Sulfur	Foliar Micronutrient	5.6L ha^{-1}			
	0-0-0-8B	Max-In® Boron	Foliar Micronutrient	1.4L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			
15	Indolebutyric acid, gibberellic acid, and kinetin	Ascend® SL	Plant Growth Regulator	469ml ha^{-1}			
	0-0-1	Toggle®	Seaweed Extract	2.8L ha^{-1}			
	0-0-19-13S	Max-In® Sulfur	Foliar Micronutrient	5.6L ha^{-1}			
	0-0-0-8B	Max-In® Boron	Foliar Micronutrient	1.4L ha^{-1}			
	Lambda-cyhalothrin	Grizzly® Too	Insecticide	134ml ha^{-1}			

† fertilizer analysis provided in percent N, P_2O_5, K_2O

‡ sulfur

¥ boron

Table 3. Analysis of variance for all examined variables, with location, 2018 alfalfa study.

	Factor analyzed										
Factor	Yield	RFQ*	ADF@	ADL#	CP$	NDF^	NDFD&	IVdOM†	ivTDM††	Ash±	TDN¶
	P > F										
Location	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Trt	0.71	0.88	0.99	0.99	0.95	0.99	0.99	0.96	0.88	0.98	0.99
Loc x Trt	0.92	0.77	0.99	0.99	0.98	0.98	0.99	0.95	0.85	0.99	0.98

* Relative Feed Quality

@ Acid Detergent Fiber

Acid Detergent Lignin

$ Crude Protein

^ Neutral Detergent Fiber

& Neutral Detergent Fiber Digestibility

† organic matter

†† total dry matter

± Ash

¶ Total Digestible Nutrients

Table 4. Analysis of variance of studied factors, Illinois (IL) location, June Cutting, 2018.

	Factor analyzed for June Cut										
Factor	Yield	RFQ[*]	ADF[@]	ADL[#]	CP[$]	NDF[^]	NDFD[&]	IVdOM[†]	ivTDM[††]	Ash[±]	TDN[¶]
	P > F										
Trt	0.51	0.97	0.96	0.99	0.92	0.95	0.95	0.99	0.96	0.92	0.98
Rep	0.34	0.99	0.96	0.92	0.91	0.87	0.35	0.99	0.86	0.03	0.95

* Relative Feed Quality

@ Acid Detergent Fiber

Acid Detergent Lignin

$ Crude Protein

^ Neutral Detergent Fiber

& Neutral Detergent Fiber Digestibility

† organic matter

†† total dry matter

± Ash

¶ Total Digestible Nutrients

Table 5. Analysis of variance of studied factors, Illinois (IL) location, July Cutting, 2018.

	Factor analyzed for July Cut										
Factor	Yield	RFQ[*]	ADF[@]	ADL[#]	CP[$]	NDF[^]	NDFD[&]	IVdOM[†]	ivTDM[††]	Ash[±]	TDN[¶]
	P > F										
Trt	0.38	0.64	0.82	0.56	0.77	0.62	0.60	0.70	0.73	0.27	0.65
Rep	0.02	0.28	0.26	0.43	0.49	0.29	0.22	0.29	0.22	0.02	0.23

* Relative Feed Quality
@ Acid Detergent Fiber
\# Acid Detergent Lignin
$ Crude Protein
^ Neutral Detergent Fiber
& Neutral Detergent Fiber Digestibility
† organic matter
†† total dry matter
± Ash
¶ Total Digestible Nutrients

Table 6. Analysis of variance of studied factors, Purina Farms (PF) location, June Cutting, 2018.

	Factor analyzed for June Cut										
Factor	Yield	RFQ*	ADF@	ADL#	CP$	NDF^	NDFD&	IVdOM†	ivTDM††	Ash±	TDN¶
	P > F										
Trt	0.29	0.59	0.71	0.78	0.46	0.75	0.43	0.56	0.44	0.41	0.58
Rep	0.0001	0.18	0.34	0.06	0.46	0.46	0.02	0.04	0.06	0.50	0.17

* Relative Feed Quality
@ Acid Detergent Fiber
# Acid Detergent Lignin
$ Crude Protein
^ Neutral Detergent Fiber
& Neutral Detergent Fiber Digestibility
† organic matter
†† total dry matter
± Ash
¶ Total Digestible Nutrients

Table 7. Analysis of variance of studied factors, Purina Farms (PF) location, July Cutting, 2018.

	Factor analyzed for July Cut										
Factor	Yield	RFQ*	ADF@	ADL#	CP$	NDF^	NDFD&	IVdOM†	ivTDM††	Ash±	TDN¶
	P > F										
Trt	0.04	0.03	0.29	0.04	0.06	0.03	0.14	0.03	0.02	0.07	0.06
Rep	0.03	0.46	0.02	0.26	0.60	0.42	0.74	0.46	0.53	0.80	0.54

* Relative Feed Quality
@ Acid Detergent Fiber
# Acid Detergent Lignin
$ Crude Protein
^ Neutral Detergent Fiber
& Neutral Detergent Fiber Digestibility
† organic matter
†† total dry matter
± Ash
¶ Total Digestible Nutrients

Table 8: Yield and Significant Quality Factors of reduced-lignin alfalfa (HarvaTron) at Purina Farms, July Cut 2018.

	Treatment Description	Yield (Mg ha^{-1})	RFQ	ADL	NDF	IVdOM	ivTDM
1	Control	2.87 bc	207 abc	4.88 abc	33.1 abc	73.6 abc	82.4 abcd
2	Max-in Sulfur† and Max-In Boron††	3.05 abc	199 abc	5.06 abc	34.1 abc	72.7 abc	81.6 abcd
3	Ascend SL± – IBA, GA, K	2..85 bc	225 a	4.64 c	31.0 c	74.8 ab	84.0 a
4	Toggle ¶- Seaweed	3.03 abc	205 abc	4.88 abc	32.7 abc	74.0 abc	82.3 abcd
5	Kriss^ – Protein hydrolysate	3.43 abc	178 bc	5.20 abc	36.2 ab	71.2 bc	79.5 cd
6	AGM16013# – IBA, GA, K	2.91 bc	175 c	5.58 a	37.0 a	70.6 c	78.8 d
7	AGM16011& – IBA, GA, K	3.03 abc	223 a	4.82 bc	31.1 c	74.6 ab	84.0 a
8	Ascend SL± + Toggle¶	3.79 b	186 abc	5.40 ab	35.5 abc	71.6 abc	79.8 bcd
9	Ascend SL± + Kriss	2.60 c	191 abc	5.12 abc	35.0 abc	72.1 abc	81.0 abcd
10	Ascend SL± + Max-In Sulfur† and Max-In Boron††	2.67 c	219 ab	4.84 bc	31.2 c	74.5 ab	82.9 abc
11	Kriss^ + Max-In Sulfur† and Max-In Boron††	3.05 abc	221 a	4.54 c	31.4 bc	74.2 ab	83.6 ab
12	Toggle¶ + Max-In Sulfur† and Max-In Boron††	3.92 a	177 c	5.45 ab	36.4 a	70.7 c	79.6 bcd
13	AGM16013# + Max-In Sulfur† and Max-In Boron††	2.87 bc	204 abc	4.90 abc	33.1 abc	73.5 abc	82.1 abcd
14	AGM 16011& + Max-In Sulfur† and Max-In Boron††	2.76 c	201 abc	4.96 abc	33.8 abc	73.7 abc	82.4 abcd
15	Ascend SL± + Toggle¶ + Max-In Sulfur† and Max-In Boron††	3.45 abc	225 a	4.64 c	31.0 c	75.0 a	84.1 a

† contains 13% sulfur derived from potassium thiosulfate
†† contains 8%B derived from boric acid
± contains 0.045% indolebutyric acid, 0.03% gibberellic acid and 0.09% kinetin
¶ contains Ascophyllum nodosum
^ contains 5% nitrogen, 8% phosphate, 4% potash, derived from urea, monoammonium phosphate, potassium nitrate, respectively, and yeast extract.
# contains indolebutyric acid, gibberellic acid and kinetin in proprietary experimental amounts
& contains gibberellic acid

Table 9: Yield of reduced-lignin alfalfa (HarvaTron) as affected by location and treatments, 2018.

Trt	Treatment Description	Location		
		IL (2 cuttings)	PF (2 cuttings)	West Salem (3 cuttings)
		Total Yield (Mg ha^{-1})		
1	Control	8.34 a	7.06 a	11.84 a
2	Max-in Sulfur[†] and Max-In Boron[††]	8.46 a	6.47 a	11.60 a
3	Ascend SL[±] – IBA, GA, K	8.60 a	8.60 a	11.46 a
4	Toggle[¶] - Seaweed	8.28 a	6.85 a	11.48 a
5	Kriss[^] – Protein hydrolysate	7.96 a	6.80 a	11.12 a
6	AGM16013[#] – IBA, GA, K	7.38 a	7.17 a	11.23 a
7	AGM16011[&] – GA	8.02 a	8.28 a	11.31 a
8	Ascend SL[±] + Toggle[¶]	7.56 a	7.84 a	10.88 a
9	Ascend SL[±] + Kriss[^]	8.08 a	7.63 a	10.65 a
10	Ascend SL[±] + Max-In Sulfur[†] and Max-In Boron[††]	8.18 a	5.63 a	11.09 a
11	Kriss[^] + Max-In Sulfur[†] and Max-In Boron[††]	8.36 a	6.56 a	11.12 a
12	Toggle[¶] + Max-In Sulfur[†] and Max-In Boron[††]	7.99 a	8.47 a	10.86 a
13	AGM16013[#] + Max-In Sulfur[†] and Max-In Boron[††]	8.65 a	6.77 a	10.95 a
14	AGM 16011[&] + Max-In Sulfur[†] and Max-In Boron[††]	8.14 a	6.92 a	11.11 a
15	Ascend SL[±] + Toggle[¶] + Max-In Sulfur[†] and Max-In Boron[†	7.52 a	6.59 a	10.65 a

[†] contains 13% sulfur derived from potassium thiosulfate

[††] contains 8%B derived from boric acid

[±] contains 0.045% indolebutyric acid, 0.03% gibberellic acid and 0.09% kinetin

[¶] contains Ascophyllum nodosum

[^] contains 5% nitrogen, 8% phosphate, 4% potash, derived from urea, monoammonium phosphate, potassium nitrate, respectively, and yeast extract.

[#] contains indolebutyric acid, gibberellic acid and kinetin in proprietary experimental amounts

[&] contains gibberellic acid

Table 10. Analysis of variance of studied factors, West Salem (WS) location, June Cutting, 2018.

	Factor analyzed for June Cut										
Factor	Yield	RFQ*	ADF@	ADL#	CP$	NDF^	NDFD&	IVdOM†	ivTDM††	Ash±	TDN¶
	P > F										
Trt	0.14	0.63	0.55	0.72	0.18	0.66	0.75	0.42	0.59	0.35	0.54
Rep	0.0001	0.46	0.52	0.81	0.08	0.53	0.45	0.51	0.33	0.30	0.40

* Relative Feed Quality
@ Acid Detergent Fiber
# Acid Detergent Lignin
$ Crude Protein
^ Neutral Detergent Fiber
& Neutral Detergent Fiber Digestibility
† organic matter
†† total dry matter
± Ash
¶ Total Digestible Nutrients

Table 11. Analysis of variance of studied factors, West Salem (WS) location, July Cutting, 2018.

	Factor analyzed for July Cut										
Factor	Yield	RFQ*	ADF@	ADL#	CP$	NDF^	NDFD&	IVdOM†	ivTDM††	Ash±	TDN¶
	P > F										
Trt	0.50	0.64	0.57	0.49	0.37	0.60	0.10	0.82	0.69	0.07	0.61
Rep	0.05	0.06	0.05	0.13	0.13	0.05	0.70	0.10	0.14	0.32	0.05

* Relative Feed Quality
@ Acid Detergent Fiber
# Acid Detergent Lignin
$ Crude Protein
^ Neutral Detergent Fiber
& Neutral Detergent Fiber Digestibility
† organic matter
†† total dry matter
± Ash
¶ Total Digestible Nutrients

Table 12. Analysis of variance of studied factors, West Salem (WS) location, August Cutting, 2018.

	Factor analyzed for August Cut										
Factor	Yield	RFQ*	ADF@	ADL#	CP$	NDF^	NDFD&	IVdOM†	ivTDM††	Ash±	TDN¶
	P > F										
Trt	0.0037	0.86	0.95	0.48	0.83	0.93	0.82	0.83	0.92	0.68	0.90
Rep	0.0021	0.66	0.67	0.37	0.83	0.71	0.80	0.75	0.82	0.23	0.71

* Relative Feed Quality
@ Acid Detergent Fiber
# Acid Detergent Lignin
$ Crude Protein
^ Neutral Detergent Fiber
& Neutral Detergent Fiber Digestibility
† organic matter
†† total dry matter
± Ash
¶ Total Digestible Nutrients

Table 13: Yield of reduced-lignin alfalfa (HarvaTron) at West Salem, August Cut, 2018.

Trt	Treatment Description	Yield (Mg ha^{-1})
1	Control	3.18 a
2	Max-in Sulfur[†] and Max-In Boron[††]	3.07 abc
3	Ascend SL[±] – IBA, GA, K	3.07 abc
4	Toggle[¶] - Seaweed	3.16 a
5	Kriss[^] – Protein hydrolysate	3.03 abcd
6	AGM16013[#] – IBA, GA, K	3.14 a
7	AGM16011[#] – IBA, GA, K	3.14 a
8	Ascend SL[±] + Toggle	3.09 ab
9	Ascend SL[±] + Kriss	2.89 bcd
10	Ascend SL[±] + Max-In Sulfur[†] and Max-In Boron[††]	3.05 abc
11	Kriss[^] + Max-In Sulfur[†] and Max-In Boron[††]	3.09 ab
12	Toggle[¶] + Max-In Sulfur[†] and Max-In Boron[††]	2.87 cd
13	AGM16013[#] + Max-In Sulfur[†] and Max-In Boron[††]	2.85 d
14	AGM 16011[#] + Max-In Sulfur[†] and Max-In Boron[††]	3.00 abcd
15	Ascend SL[±] + Toggle[¶] + Max-In Sulfur[†] and Max-In Boron[††]	2.98 abcd

† contains 13% sulfur derived from potassium thiosulfate

†† contains 8%B derived from boric acid

± contains 0.045% indolebutyric acid, 0.03% gibberellic acid and 0.09% kinetin

¶ contains Ascophyllum nodosum

^ contains 5% nitrogen, 8% phosphate, 4% potash, derived from urea, monoammonium phosphate, potassium nitrate, respectively, and yeast extract.

contains indolebutyric acid, gibberellic acid and kinetin in proprietary experimental amounts

Table 14: Quality of reduced-lignin alfalfa (HarvaTron), averaged over location and cuttings, 2018.

TRT	Treatment Description	RFQ	ADF	ADL	CP	NDF	NDFD	IVdOM	ivTDM	Ash
1	Control	188.57 a	30.36 a	5.05 a	21.86 a	35.50 a	46.92 a	73.01 a	79.99 a	7.23 a
2	Max-In Sulfur† + Max-In Boron††	190.24 a	30.28 a	4.98 a	22.03 a	35.28 a	47.07 a	72.94 a	80.02 a	7.35 a
3	Ascend SL± – IBA, GA, K	194.00 a	30.07 a	4.97 a	21.91 a	35.01 a	47.22 a	72.99 a	80.22 a	7.28 a
4	Toggle¶ - Seaweed	188.99 a	30.39 a	5.07 a	21.91 a	35.41 a	46.51 a	72.94 a	79.85 a	7.23 a
5	Kriss^ – Protein hydrolysate	184.62 a	30.57 a	5.07 a	21.93 a	35.81 a	46.39 a	72.60 a	79.55 a	7.07 a
6	AGM16013# – IBA, GA, K	185.76 a	30.70 a	5.08 a	21.57 a	35.99 a	46.55 a	72.30 a	79.45 a	7.27 a
7	AGM16011& – IBA, GA, K	192.34 a	30.04 a	5.00 a	21.83 a	34.95 a	47.25 a	73.17 a	80.33 a	7.21 a
8	Ascend SL± + Toggle¶	188.74 a	30.30 a	5.06 a	21.88 a	35.42 a	46.61 a	72.77 a	79.84 a	7.30 a
9	Ascend SL± + Kriss^	184.83 a	30.64 a	5.12 a	21.48 a	35.82 a	46.31 a	72.48 a	79.42 a	7.11 a
10	Ascend SL± + Max-In Sulfur† and Max-In Boron††	194.15 a	29.65 a	4.97 a	21.94 a	34.67 a	46.86 a	73.12 a	80.23 a	7.23 a
11	Kriss^ + Max-In Sulfur† and Max-In Boron††	190.21 a	30.14 a	4.96 a	21.90 a	35.26 a	46.99 a	72.74 a	80.06 a	7.29 a
12	Toggle¶ + Max-In Sulfur† and Max-In Boron††	180.00 a	31.05 a	5.22 a	21.49 a	36.36 a	45.53 a	71.91 a	78.91 a	7.06 a
13	AGM16013# + Max-In Sulfur† and Max-In Boron††	188.51 a	30.20 a	5.07 a	21.93 a	35.28 a	46.80 a	72.80 a	79.96 a	7.24 a
14	AGM 16011& + Max-In Sulfur† and Max-In Boron††	188.12 a	30.26 a	4.97 a	21.64 a	35.51 a	46.98 a	72.98 a	79.99 a	7.27 a
15	Ascend SL± + Toggle¶ + Max-In Sulfur† and Max-In Boron††	193.50 a	30.07 a	4.98 a	22.25 a	34.97 a	47.44 a	73.18 a	80.41 a	7.42 a

† contains 13% sulfur derived from potassium thiosulfate

†† contains 8%B derived from boric acid

± contains 0.045% indolebutyric acid, 0.03% gibberellic acid and 0.09% kinetin

¶ contains Ascophyllum nodosum

^ contains 5% nitrogen, 8% phosphate, 4% potash, derived from urea, monoammonium phosphate, potassium nitrate, respectively, and yeast extract.

contains indolebutyric acid, gibberellic acid and kinetin in proprietary experimental amounts

& contains gibberellic acid

Lignin Biosynthesis Pathway

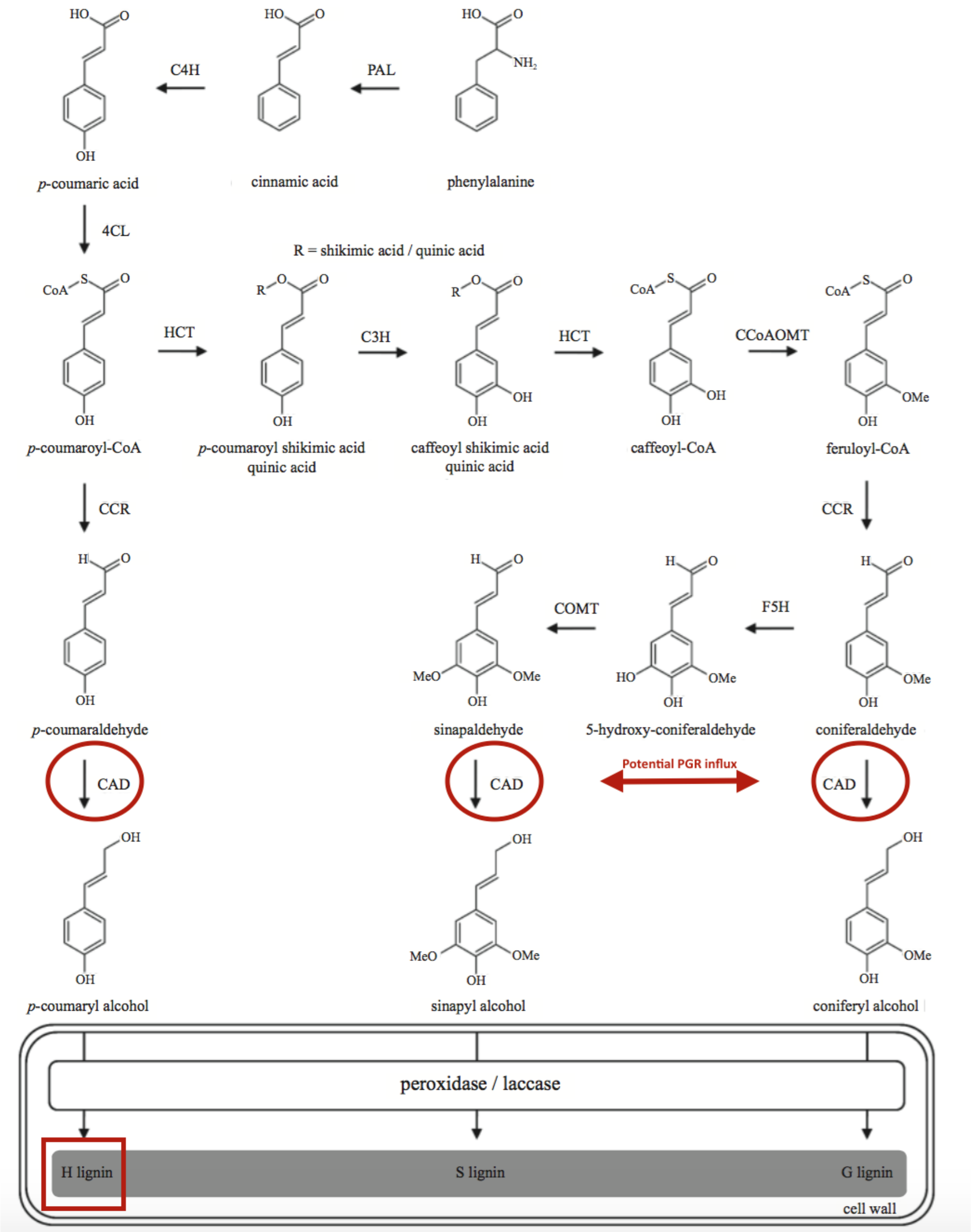

Fig. 1 Adapted from Cesarino, *et al.*, 2012, *Figure 1* shows the lignin biosynthesis pathway. The red double arrow indicates where plant hormones/PGRs could bypass the COMT and CCOMT knockout. The red circles indicate the enzyme of interest to induce a gene knockout for qualitative trait improvement of reduced lignin in alfalfa.