Project 9028 Final Report
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Project Title:

The Quantification and Identification of Hay Dust as it Relates to Hay Quality

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Executive Summary

Horse owners have a great concern about dust in hay and its potential to cause allergic airway disease in horses, commonly known as heaves. Hay producers are quite concerned by negative feedback from horse owners when hay is considered dusty. This project initiated an investigation into the biological components of hay dust and the affect of hay production practices on hay quality from a dust standpoint.

A summer student was hired under the Ontario government Summer Experience Program. A search of the literature revealed very little additional information on hay dust evaluation. Extensive literature was found on stable hygiene and horse health as it relates to dust.

Hay samples were collected by the summer student during the 2007 harvest season. The samples were tested using commercial laboratory methodology (Stomacher bags and Seward Stomacher laboratory blender). Equipment and techniques to extract dislodgeable particles from hay were developed. Preliminary extractions of dust particles from hay samples onto three devices were completed.

A surprising finding was the presence of a common alfalfa fungal pathogen (*Phoma* sp.) on the majority of Petri plates using the traditional commercial laboratory techniques. This pathogen may be responsible for an overestimation of the dustiness of hay using traditional laboratory analyses. Future research will focus on evaluation and validation of our experimental methodology in comparison to traditional laboratory methods. The hay samples collected in the past two harvest years will be used for these comparisons. Collaborative studies have been initiated to evaluate the significance of the prevalent alfalfa pathogen, *Phoma* sp., on the respiratory health of horses.

The preliminary phase of this project progressed well. All objectives were completed using part-time technical assistance and in-stream, graduate-student assistance with experimental protocols. The surprising finding of a suspected fungal plant pathogen, *Phoma* sp., will be directing collaborative research into the immunogenic response of horses to this common plant pathogen.

All in-kind funding was received. Additional in-kind financial support for hiring a summer student to collect the hay samples was provided from the Ontario government Summer Experience Program.

Introduction

Horse owners are very concerned with dust in hay intended for use with horses. Any sign of dust leads to panic, rejection of deliveries of hay, and ill feelings between hay producers and horse owners. The panic is a direct fear of horses developing allergic airways disease, chronic obstructive pulmonary disease (COPD), commonly referred to as heaves, in response to dust in hay.

The particles that we observe as dust in hay can come from a number of sources, both pre-harvest and post harvest.

Pre-harvest dust particles can be caused by:

- Soil particles incorporated into the hay, either by raking, rain splash or proximity to dusty roads and fields.
- Trichomes (hairs, epidermal outgrowths, scales) are present on the surfaces of growing forages. Plants, such as red clover, have higher numbers of hairs than other plants.
- Mites - Storage mites feed on a variety of substances and can be found in many different products, such as grain, flour, hay and straw, but also in house dust samples.
- Leaf shatter that occurs under extremely dry harvest conditions. Equipment and handling result in pulverization of leaves. This material easily becomes airborne during horse feeding.
- Mold spores that are associated with airborne concentrations of spores and fungal plant pathogens. These fungi can be endophytes or saprophytes growing in or on living plants. Saprophytic fungal growth develops on forages during humid and wet conditions pre-cutting and/or between cutting and baling when drying is delayed in the windrows. Most of this increased fungal growth is caused by fungi that prefer ambient temperatures (e.g. 20-22^{o}C) for growth and development (mesophiles).

Post harvest dust particles can be caused by;

- Fungi growing on stored hay. Higher spore counts will be associated with hay stored at moisture levels greater than 14 % (less than 86 % dry matter). Most of this increased growth is associated with fungi that prefer temperatures around 37^{o}C (thermophiles).

This project is Phase 1 of a multi-year project to investigate the “Quantification and Identification of Hay Dust as it Relates to Hay Quality”.

The overall objectives of this multi-year project are to:

1. Develop a standardized method for collecting and quantifying “dislodgeable” particles in hay;
2. Quantify the dislodgeable particles into size ranges;

3. Identify the sources of dislodgeable particles (e.g., inorganic or organic material, mites, mold spores, mold hyphae), which are in the respirable particle size for horses;
4. Validate the use of a particle counter for determining dust-particle size in hay against other laboratory methods (e.g., colony forming units from agar plate mould counts); and
5. Communicate these findings through industry talks and publications.

Project Results and Milestones

1. Summer Student pilot project – May 1 to Aug 10, 2007

A summer student was hired under the Ontario government Summer Experience Program. A cooperator farm was located, and hay samples were collected under various harvest and storage conditions (trials) to use in the laboratory Phase 2 of the project. Pre- harvest production practices and environmental data related to the harvest and storage conditions (trials) were collected. Mows/bales of hay were monitored every 30 minutes for temperature fluctuations from day 1 to day 30 post harvest, using data loggers (Hobo, outdoor/industrial 4-channel data loggers, Onset Computer Corporation). Ambient temperature and humidity within the barn was recorded concurrently. Representative samples from mows/bales of hay were taken weekly for evaluation using a hay core sampler. Samples were frozen for Phase 2 of the project.

2. Literature Search – Summer & Fall 2007

A search of the literature on research pertaining to the evaluation of dust in hay was completed. Very little additional information on evaluating or quantifying dust in hay was found. The bulk of research on the clinical syndrome referred to as heaves is related to stable hygiene and horse health or disease treatment. Searches of the literature will continue. The relevance of the research will be evaluated.

3. Evaluation of current commercial laboratory methodologies, September 2007

Gelda Scientific is a commercial laboratory that provides microbiological and chemical testing services to the pharmaceutical, food, beverage, cosmetic, environmental and agricultural industries. It is the principle diagnostic laboratory in Ontario for evaluating hay samples for mold content. Gelda Scientific has developed a protocol for assessing hay quality in previous years. This protocol processes samples of hay using a laboratory stomacher blender that breaks down the sample into a liquid slurry that is then serially diluted and plated on microbiological media, such as rose-bengal-agar (RBA). Exposed plates are incubated at selected temperatures for 2-3 days, and the number of colonies developing on each plate are counted and expressed as the number of colony-forming-units per gram (CFU/g) of hay sample.

All hay samples from all treatments were collected and submitted to Gelda Scientific for processing using their accepted protocol. These samples were considered to be control or standard results for comparisons with other methods.

The current commercial laboratory testing of hay uses the following methodology:

- 10 grams of hay are placed in a Stomacher bag with 100 mL of buffered saline and PDA.
- The bag is placed in a Seward Stomacher lab blender for 30 seconds. The round-bottom, Stomacher-lab-blender bags mold the bag contents into a tubular ring. During operation the paddles crush the sample and circulate the suspended debris and diluent. In this process,

the diluent and sample are extruded under pressure past the island baffle, and are vigorously stirred from top to bottom. This unique paddle action extracts the organisms into suspension.
- One mL of the samples is extracted, and ten-fold serial dilutions are prepared.
- The samples are plated onto Petri plates containing approx. 20 mL of Rose Bengal Agar (RBA) medium, and incubated at 22^{o}C and 37^{o}C for 2-3 days.
- Mold counts are manually performed and recorded.

When assessing several of the hay samples using the current laboratory methodologies, a suspected plant pathogen, a *Phoma* species, was found to be a primary contributor to spore/mold growth on the RBA culture plates. It is postulated that the maceration of samples, using the Stomacher bag and blender, extracts mold spores from the hay that normally would be imbedded within a mucilaginous matrix inside the spore-producing structure of this fungus (e.g. pycnidia). Typically, these spores are considered to be "wet-spored" fungi and are primarily released during periods of rain that cause the mucilaginous matrix to swell and release the spores from the pycnidia. Therefore, these spores would normally not be respired as a component of atmospheric aerosols or would be filtered out along with other large particles within the upper airways of the horse.

4. Hay tumbler - May 2007 and ongoing

In the course of the project, we realized that it was important to develop a method of extracting particles from hay in a representative method that would mimic the exposure of horses to these particles. Therefore, a "hay tumbler" was developed. The hay tumbler gently turns and/or tosses a sub-sample of the hay at 40 RPM. Mild vacuum pressures are used to extract an air sample containing dislodgeable materials (e.g., aerosols) from the core of the tumbler. This air stream is then assessed using various devices for evaluation. Photographs of the tumbler and the three devices that we are currently assessing are appended to this report.

5. Development of a standardized method for collecting and quantifying "dislodgeable" particles in hay – May 2007 and ongoing

The key to collecting "dislodgeable" particles from hay was the development of the hay tumbler, which could deliver aerosol particles through vacuum to various devices. We are currently testing three devices, including;

- **N6 (Anderson) Single Stage Viable Impactor**

  The single stage, N6, microbial impactor was specifically designed to meet international regulatory specifications for bioaerosol sampling protocols. This inertial impactor obtains a sharp cut-off diameter of 0.65 μm, and deposits collected particles directly onto microbiological media. An attachment was designed to adapt the N6 (Anderson) Single Stage Viable Impactor to the hay tumbler.

  Hay samples (e.g., 30 g) were processed for 30, 60, and 120 seconds at 15 L/min vacuum, and the airstream containing particles of the desired particle range was impacted onto potato-dextrose agar (PDA) or acidified PDA (to reduce bacterial growth) media. Exposed plates were incubated for 2-3 days at 20-22^{o}C and 37^{o}C, and the number of colonies developing on each plate was counted and expressed as the number of colony-forming-units per g (CFU/g) of hay sample. Identification of the dominant fungi types was recorded.

- **Marple 290 Personal Cascade Impactor**

The Marple 290 Personal Cascade Impactor is a precision cascade impactor worn by personnel or animals to provide complete and accurate aerodynamic particle size distributions of the particulate in their environment. It has a flow rate of 2 Lpm and is intended to be utilized with a personal sampling pump. Collected bioaerosols enter the device and are accelerated through six radial slots in the first impactor stage. Particles larger than the cut-point of the stage impact on the pre-cut collection filter. Airstream flows through the narrower slots on the second impactor stage, smaller particles impact on the second collection filter and so on throughout the eight-stage impactor. Filters are weighed prior to and after a sampling. A weight increase of the filters is the mass of the particles.

Test runs using this device have been completed. Phase 2 of the multi-year project will process all hay samples (e.g., 30 g) for varying periods of time (e.g., 30 sec to 5 min) at 15 L/min vacuum, and the filters then weighed to determine the total amount of particulate matter in the eight stages or size ranges. Sections of filters will also be used for serial dilution and plating of particles onto media. The media and evaluation of colonies will be as previously described.

Zefon Bio-Pump and Air-O-Cell Cassette

The Zefon Bio-Pump and Air-O-Cell Cassette is a sampling technology designed for the rapid collection and analysis of a wide range of airborne aerosols. These include fungal spores, pollen, insect parts, skin-cell fragments, fibers, and inorganic particulates. Samples are collected onto gel-coated microscope slides within the cassettes, which are then examined in a microscope. An attachment was designed to adapt the Zefon Bio-Pump and Air-O-Cell Cassettes to the hay tumbler.

Hay samples (e.g., 30 g) were processed for 30, 60, and 120 seconds at 15 L/min vacuum, and the air-o-cell cassettes were used to collect aerosol samples from the tumbler. Individual cassettes were assessed by EMC Scientific, an independent environmental microbiology laboratory specializing in analysis of building-related environmental microbiology samples. The samples were examined microscopically at 100x and 400x to estimate the number of fungal spores and other identifiable materials within the collected samples. Results were tabulated as the total number of fungal spores/g sample. All samples were repeated at least once.

Significance of the Project to Agriculture

One of the big surprises in the research was the predominance of a *Phoma* sp. as the primary contributor to spore/mold growth on the RBA cultures in our samples, using the traditional method of analysis. We believe that this fungus is the causal agent of the alfalfa disease, spring black stem, caused by *Phoma medicaginis* var. *medicaginis*. This fungus is a common pathogen of alfalfa and grows well in the spring on first-cut alfalfa. The fungus produces small fruiting bodies (e.g., pycnidia), which survive during the winter and summer in alfalfa stubble. During periods of cool (18-24^{o}C), wet weather, spores ooze from these tiny fruiting bodies and are splashed to young leaves and stems. Plant breeders have not been able to develop varieties of alfalfa that are resistant to this pathogen.

The finding of *Phoma* sp. as the primary source of high spore counts redirects our thinking as to;

- The current laboratory methodology for processing samples. For example, do we need to separate plant pathogens from hay-storage-associated molds using different handling techniques or selective media?
- The possible role of plant pathogens in allergic airways disease in horses.
- The effect on the growing and marketing of hay should *Phoma* sp. be a contributor to airways disease in horses.

Due to the possible importance of *Phoma* spp. in hay, isolates of this fungus are being evaluated for possible allergenicity in horses.

Project Expenditures

A full accounting of the project's financial status has been submitted by the Office of Research, University of Guelph, including a listing of the other funding sources.

Communications Plan

Winter 2007: Presentations on hay and hay mixes will be included in a number of updates for seed distributors, feed dealers and horse owners.

June 2009: Presentation at the next biannual meeting of Equine Science conference.