Submission concerning EPA application number APP201774, by AgResearch - Grasslanz to release potentially toxic *Neotyphodium* fungi into the New Zealand cereal grains market.

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If the application by AgResearch-Grasslanz is approved by the Authority this will have inevitable implications for the citizens of New Zealand, their pets and their farm livestock, and could have serious consequences for New Zealand exports of human foods and animal feedstuffs. No other country is producing cereal grains (or products made from these cereal grains) that contain endophyte insecticidal alkaloids, why would NZ choose to contaminate its cereal grains with these compounds?

The cereal grains grown in NZ, and throughout the world, are endophyte free consequently endophyte alkaloid free. AgResearch-Grasslanz is applying to release wheat, barley, rye, triticale, and oats, seed lines into the market place that contain endophyte alkaloids. The insertion of endophyte into a plant is akin to altering its genetics because it alters the plants gene switches hence alters the amounts of specific types of chemical that the plant subsequently produces. Alkaloids that were present in only small amounts in "wild" endophyte infected plants for example can suddenly be produced in very large amounts in plants inoculated with a "selected strain" of an endophyte. We have so far seen this adverse outcome in three AgResearch-Grasslanz products, namely: the Perennial Ryegrass plus Endosafe endophyte combination (found after release, to produce toxicity causing ergovaline alkaloid levels which were higher than that of ' wild' endophyte), the Perennial Ryegrass plus AR37 endophyte combination (found after release, to produce toxicity causing janthitrem alkaloids capable of causing outbreaks of ryegrass staggers in livestock more serious than those caused by 'wild' endophyte), and most recently the Mediterranean tall fescue grass plus Max P (or sometimes Max Q) endophyte combination (found after release to produce a new form of lethal toxicity in horses, most likely due to the very high levels of a particular loline compound that are produced by this combination). When the AgResearch-Grasslanz new selected endophyte containing cereal grains are harvested and processed, the flour, bran or clean grain produced will end up in the diets of all NZ humans, their pets, and their farm livestock. Endophyte alkaloids will in this way end up in your breakfast cereal, your bread sandwich at lunch, your cakes or biscuits at afternoon tea and

your pasta and pudding at dinner. Endophyte alkaloids will also end up in the processed dog and cat foods sold as pet food in the supermarket, in the various dry feed concentrates sold to horse owners, in the feedstuffs sold for pigs, milking cows and goats, stud sheep, cattle, deer, alpacas and poultry. They will also be in the cereal fodders grazed by all of these livestock species and they will be in the cereal hay products that NZ currently exports to racehorse owners, dairy farms and feedlot operators throughout south-east Asia.

The adverse risk most inadequately discussed by the applicants in their proposal, is the potential toxicity of the endophyte alkaloid insecticides that they are proposing to contaminate NZ cereal grains with. The applicants repeatedly call these substances "non-toxic" and "safe", this is misleading, these substances are more accurately described as 'potentially toxic' since no comprehensive animal studies have yet been conducted to ascertain their toxic dose level in each of the common animal species that are to be exposed to them. The literature frequently says that various endophyte alkaloids are non-toxic or safe and then gives as a reference a piece of work in which this assumption was stated but never tested, all the new reference does is to lead the reader back to yet another reference that also says the substances are non-toxic or safe, but presents no experimental data to verify this. The applicants in their current proposal are guilty of this same misleading reference usage.

To understand the complexity of this adverse risk it is necessary to understand that toxin sensitivity varies between animal species and this sensitivity is usually dose rate dependant. Most alkaloids will exert toxicity in most species if a sufficiently high enough oral dose is administered. Once this dose has been established it becomes possible to calculate how much of the alkaloid is produced by the plant plus endophyte combination and how much of the plant will be eaten each day by a particular animal species, from this a conclusion can be drawn as to the maximum likely dose rate of alkaloid that will be ingested and how this relates to the known toxic dose. In this way it will become obvious if toxicity is a risk or not. If the toxic dose for each alkaloid were to be established in test groups of animals, representative of each major animal species, then it would be possible to reasonably conclude what the human risk was likely to be. There are five minimum test groups required, namely the sheep, the horse, the pig, the dog and the chook. These groups cover the major digestive systems namely, ruminant, monogastric herbivore, monogastric omnivore, monogastric carnivore and avian. Humans are monogastric omnivores consequently the test results for pigs would offer a reasonable guide as to the human toxicity risk. Rodent (mice, rats etc) toxicity data is frequently used internationally for toxicity determinations but it provides little or no guide as to the real toxicity risk posed to other mammalian species because rodents are notoriously tolerant of toxins. AgResearch-Grasslanz is in a better position than anyone else to conduct this fundamental toxicity research and they have the most to gain by it. It should be a prerequisite for the type of EPA application they are

making. Presumably they have avoided carrying out these more comprehensive toxicity studies because, no authority has so far required them to, they are expensive studies to conduct, they do not produce any direct commercial dollar returns to the company, and if the results indicate potential toxicity problems then this could prevent AgResearch-Grasslanz from pursuing the development and release of a number of new products. Rather than constantly demanding that other people demonstrate that the endophyte alkaloids are potentially toxic, and meanwhile stating that they are non-toxic and safe, it would be much more reasonable to expect AgResearch-Grasslanz itself to establish beyond any doubt what the toxic dose level is for each alkaloid in each major animal species. After all it is AgResearch-Grasslanz that is sending these endophyte products into the market place for their own commercial purposes.

To highlight the inadequacy of the adverse risk toxicity data provided in AgResearch-Grasslanz's application it is necessary to first consider an overview of the endophyte alkaloid production principal and then to look at the individual groups of alkaloids that are the subject of the AgResearch-Grasslanz application. Endophytes produce a limited range of alkaloids namely:

1. Ergopeptines (ergot alkaloids), typically ergovaline and ergotamine, but also the related clavines and ergines (lysergic acid amide).

2. Lolines (pyrrolizidine alkaloids), typically formyl-loline, acetyl-loline and acetyl-norloline.

3. Pyrrolo-pyrazines usually peramine

4. Lolitrems, typically lolitrem B and paxilline, but also the related janthitrems.

When individual endophyte strains are isolated they can be selected for the different alkaloid mix that they produce, for example a strain that does not produce ergot alkaloids can be selected but as a consequence it would normally be expected to produce larger amounts of one or more of the other three alkaloid types. This is the inherent danger of using selected endophyte strain technology you run a great risk of creating new forms of animal toxicity because the new strain may be producing large amounts of a previously innocuous alkaloid. The endophyte will always compensate, selection pressure against one alkaloid will always result in automatic selection for another alkaloid. For example the Max P endophyte (also known as AR542) was selected because it does not produce ergot alkaloids or lolitrems, instead it producers large amounts of lolines and significant amounts of peramine. The second problem with using selected endophyte strains is that they interact with their plant host in unpredictable ways. For example the combination Max P plus summer active strains of tall fescue grass producers small to moderate amounts of lolines whereas the combination Max P plus winter active strains of tall fescue grass producers very large amounts of lolines.

Ergopeptines (ergot alkaloids)

AgResearch-Grasslanz have acknowledged that the ergot alkaloids are toxic, both to animals and humans and the literature is full of examples of this toxicity. However, they seek to release an endophyte strain that producers chanoclavine (a precursor of ergovaline) which they note lacks a key structural feature of ergovaline hence would not be expected to produce the same form of toxicity as ergovaline. They then say that they have no evidence that chanoclavine is toxic and therefore it must not be toxic. This is an appalling conclusion, the reality is that there has never been a comprehensive toxicity study carried out using chanoclavine and so its toxicity risk remains unknown, it will have a toxic dose rate and this must be established. Just because it is unlikely to intoxicate in the same way as ergovaline does not mean that it is unlikely to intoxicate. An endophyte that produces lots of chanoclavine may be an endophyte that is going to produce a new livestock toxicity state.

Lolines (pyrrolizidine alkaloids)

These are unusual pyrrolizidine alkaloids because a small change in their chemical structure has meant that they are not hepatotoxic (ie liver intoxicants), most pyrrolizidine alkaloids are. In 2009 I published a paper that described a new form of intoxication in horses in Australia that had ingested the combination Max P endophyte plus winter active tall fescue grass, it is called Equine Fescue Oedema. AgResearch-Grasslanz argued at the time that this must be a peculiarly Australian problem because it had not been reported in other countries where this combination was being grown. Sometime between 2009 and 2013 AgResearch-Grasslanz conducted their own horse grazing studies in New Zealand with this combination and on each occasion they found that their trial horses became intoxicated in the same way as they had in Australia. They finally agreed that the combination was toxic to horses regardless of country and so in 2013 ceased the production and marketing of this endophyte plus grass combination (of course all of their existing seed stocks had been sold into the market place by this time). The alkaloids produced by Max P plus tall fescue grass are lolines and peramine, production of the latter is similar for both winter active and summer active grass varieties. The stand out difference between winter active and summer active plants with this endophyte is that the winter active plants produce very large amounts of lolines and the summer active plants only produce small to moderate amounts. In particular the combination Max P plus tall fescue grass results in the production of one specific loline namely N-acetyl-nor-loline, and production of this alkaloid in the winter actives (being 2000 mg/kg) is at least seven times greater (Qawasmeh, Bourke, Lee et al Acta Chromatographica 2011, 4, 621-628) than it is in the summer actives (being 286 mg/kg). The summer actives are not toxic to horses whereas the winter actives are. AgResearch-Grasslanz has not presented any of this data in their current application and continue to deny that these finding are highly suggestive of a role for N-acetyl-nor-loline in this new type of horse toxicosis. Instead they suggest the cause must be an as yet unidentified alkaloid produced by the endophyte, but

after 5 years of work they still have no idea what this proposed new alkaloid is likely to be and they have made no mention of it, or the toxicity risk that it would pose, in their current application. The simplest approach that AgResearch-Grasslanz could have taken would have been to administer an appropriate oral dose of N-acetyl-nor-loline to a group of horses and see if toxicity occurred. One can only assume that they have either already done this and produced toxicity which they have not reported, or that they have refused to do this for fear that it may demonstrate loline toxicity (specifically N-acetyl-nor-loline) and this would require them to acknowledge that lolines are not safe for mammals because they are potentially toxic to some species at levels found in some endophyte grass or cereal combinations.

My 2009 publication (Bourke, Hunt and Watson, Aust Vet J 87:492-498) had indirectly illustrated that whereas an N-acetyl-nor-loline fescue plant level of 2000 mg/kg dry matter was apparently toxic to horses within 3 to 5 days of continuous grazing it was not toxic to either sheep or cattle. Assuming the livestock dry matter intake per day was 3% of body weight and the weight of a horse is 500kg, the daily feed intake would be 15kg hence 30,000 mg of N-acetyl-nor-loline per day or a total of 90,000 to 150,000 mg over 3 to 5 days. This implies toxicity for horses occurs at an N-acetyl-nor-loline oral dose of between 180 and 300 mg/kg live weight (being 60 mg/kg live weight per day) but that toxicity for sheep and cattle would require a much higher dose rate. A recent AgResearch-Grasslanz publication by Gooneratne et al in 2012 (NZ Vet J, 60:176-182) gave a daily oral dose of lolines (mixture unspecified) at 68 mg/kg live weight to six ewe lambs for six days and produced no toxic effects. This is consistent with my observations reported in 2009 and poses the question, why when AgResearch-Grasslanz were already aware of my findings did they not challenge their experimental sheep with a much higher dose rate than this, to try and ascertain what is the toxic dose for sheep? One can only conclude that they wanted an outcome that said lolines are non-toxic. In the current application AgResearch-Grasslanz have used mice as their experimental species and found that they tolerated a repeated daily ingestion rate of a mixture (not specified) of lolines at 415 mg/kg live weight. When N-acetyl-nor-loline or N-acetyl-loline were specifically administered, mice tolerated up to 2000 mg/kg live weight, but with N-formyl-loline deaths started to occur at this same dose rate. These findings would suggest that whereas horses are intolerant of lolines rodents are very tolerant of them, with sheep and cattle presumably being somewhere in between.

Pyrrolo-pyrazines (Peramine)

My 2009 publication had indicated that peramine levels of from 6 to 26 mg/kg dry matter occur in tall fescue grass inoculated with the Max P endophyte. These are low levels of alkaloid and unlikely to pose a toxicity risk. Even at a plant level of 30 mg/kg, the daily peramine intake rate

in a 500kg horse would have been less than 1 mg/kg live weight. Sheep and cattle had also grazed the same pastures without any signs of peramine toxicity hence were safe at this dose rate. Likewise AgResearch-Grasslanz in 1995 administered a peramine oral dose rate of 0.8 mg/kg live weight daily (ie 40mg to a 50kg animal) for seven days to a group of 4 wether lambs and found no toxic effects which is consistent with my observations. More recently AgResearch-Grasslanz administered peramine at 2000 mg/kg live weight orally to mice (number not specified) with no apparent toxic effects. Rodents are in general very tolerant of toxins, it would have been more meaningful if sheep or horses or pigs or dogs or poultry had been used as the test species for this much larger dose rate.

Lolitrems and janthitrems.

Selection for endophyte strains that do not produce lolitrems inevitably risks selection for strains that produce related chemical groups such as janthitrems and terpendoles. In the current application terpendole E has been nominated as a new potential toxin. A structural change in this compound compared to that of lolitrem B would indicate it is unlikely to have a tremorgenic effect at any dose rate. However the potential toxicity otherwise of this compound remains untested. AgResearch-Grasslanz have tested it in mice (number unspecified) at a dose rate of 8mg/kg live weight and found it to be "non-tremorgenic", but they did not specifically state no toxic effects at all. This compound needs to be tested in a range of animal species at higher dose rates than this to establish what its potential for toxicity is.

Conclusion Under section 36 of the NZ environmental protection act the Authority is required to decline an application to release new organisms if they are likely to cause any significant adverse effects on human health and safety, or if they are likely to cause disease in, be parasitic to, or become a disease vector for, animals. The toxicity data provided by the applicant is insufficient to support beyond a reasonable doubt either of these minimum standards. In addition, under the adverse risks section of the application the applicant has failed to declare all of the adverse toxicity risk information that either they have in their own records or that others have published on this subject in the scientific literature. The toxicity studies of AgResearch-Grasslanz are rarely published in peer reviewed scientific journals consequently they are rarely assessed by other scientists who do not have the same commercial conflict of interest that exists within AgResearch-Grasslanz. Extensive toxicity studies using endophyte alkaloids in a range of common animal species at increasing dose rates are urgently required, this company should be obliged to carry out these studies and to have their results peer reviewed and published in the mainstream scientific literature. It is in everyone's long term interests, including those of AgResearch-Grasslanz, for this to happen.