Response to Dr Christopher Bourke submission

Introduction

Releasing the fungal *Neotyphodium* endophytes, listed in the application from containment does not mean that all NZ grain will be automatically infected with or by them – the endophytes are maternally inherited and therefore under the control of the grower. These endophytes will only be found in grain of plants artificially inoculated with them or derived from artificially inoculated plants. The fungal endophytes do not move horizontally and can only be vertically transmitted i.e. through the seed. We are seeking only the release of a designated number of strains which are fully owned and controlled by Grasslanz/AgResearch. There is no intention of moving these into commercial production without extensive toxicity testing using appropriate animal species as required by regulatory authorities. This will be most effectively achieved by having the option to grow the endophytic plants out of containment. The amount of grain required to feed a horse alone would be challenging to produce from plants grown in containment.

The introduction of *Neotyphodium* alkaloids to animal feed and human food is an issue that we take most seriously as indicated by our commitment to performing toxicity testing to date. Prior to the commercial release of any cereal with endophyte we will work with the Australian and New Zealand regulatory authorities to ensure that any and all toxicology testing required is completed and the alkaloid(/s) proved safe for consumption. This specific toxicology testing cannot be completed until we have completed our field trial work and completed our selections for commercial release. Only then will we know what alkaloids will be present and in what levels. Growing the endophytic cereals out of containment will ensure the best combination for improved pest and disease resistance are obtained. It will be the whole grain which needs to be tested as this may contain more than one alkaloid which may (or may not) act synergistically.

A number of Dr Bourke's statements are not scientifically correct. This includes the contention that the "insertion of the endophyte into a plant is akin to altering its genetics because it alters the plants gene switches". A review of the scientific data generated from decades of endophyte research, covering research on a number of commercial endophyte products, as well as fundamental research on endophyte chemistry and the genetics of the interaction indicates that this is not the case (Johnson et al. 2013). The gene pathways for production of alkaloids are found in the fungus and not the host plant. The current work with cereal endophytes is identifying suites of genes responsible for secondary metabolite production in order to predict the secondary metabolic potential of different endophyte strains by correlating the presence or absence of different genes with known and unknown chemistry.

Toxicity of lolines

A point raised by Dr Bourke is whether lolines are toxic to mammals. Our application claims that the metabolites peramine and lolines raise no concerns for food safety or livestock health. The reference that suggests a causal link between mammalian toxicosis and one loline compound, N-acetyl norloline, is one involving Dr Bourke as the lead author (namely, Bourke et al. 2009). Yet the connection is not proven in this publication, it is supposition and the conclusion is that the findings warrant investigation. The case is not proven.

Loline is linked with pyrrolizidine alkaloids in Bourke's paper, and while lolines are a member of the 1-aminopyrrolizidines they do not exhibit hepatotoxicity (chemical driven liver damage) as occurs with other alkaloids that have a pyrrolizidine ring structure, e.g. alkaloids from *Senecio* species (Fu et al. 2004). Lolines are saturated amino pyrrolizidine alkaloids with an oxygen bridge between C-2 and C-7 and have been isolated from grasses. The chemistry and biology of these substances are reviewed by Bush et al. (1993). Bush et al. (1993) demonstrates the need to clearly distinguish between the saturated amino pyrrolizidine alkaloids which are not hepatotoxic and the 1,2-unsaturated pyrrolizidine alkaloids which are very significant animal and human toxins and carcinogens of plant origin.

Fu et al. (2004) concludes that in general, cattle, horses, pigs, chickens, ducks, rats, and mice resemble humans and are susceptible to unsaturated pyrrolizidine alkaloid intoxication, whereas sheep, goats, rabbits, and guinea pigs are resistant to unsaturated pyrrolizidine alkaloid toxicity, and young animals exhibit higher susceptibility than do adults (he quotes the following references - Castagnoli et al., 1997; Huan et al., 1998a; Mattocks, 1986; Prakash et al., 1999; Robertson, 1982; Stegelmeier et al., 1999; White et al., 1973). Fu et al. 2004 does not make mention of any of the loline compounds. So while Bourke postulates that horses are more susceptible to N-acetyl norloline than rats, Fu et al. (2004) concludes for toxic unsaturated pyrrolizidine alkaloids (from plant sources other than endophytic grasses) there is no difference in toxicity/susceptibility between cattle, horses, pigs, chickens, ducks, rats, mice and humans. This demonstration of similarity of response across the test animal groups, that includes rats, indicates that it might be reasonable to assume that rats are appropriate subjects for the toxicity testing of these loline compounds. As detailed in our application we have conducted toxicity testing of loline species in rats following OECD guidelines which showed no oral toxicity even at the limit dose of 2000 mg/kg.

All literature except the Bourke paper refers to loline produced by *Neotyphodium* endophytes as insecticidal and deterrent to a broad range of insects (Schardl et al. 2007; Dahlman et al. 1991) and provides no evidence of mammalian toxicity issues.

Toxicity of peramine

As indicated by the submission, peramine is a naturally occurring pyrrolopyrazine alkaloid produced by endophytes that protect the grass against insects. There is no published evidence of mammalian toxicity to this compound and we have dosed mice at the OECD limit dose of 2000 mg/kg with no adverse effects. As acknowledged in the Dr Bourke submission, peramine levels in herbage are low and horses could ingest a maximum of less than 1 mg/kg live weight/day. The dosing of a horse at 2000 mg/kg, as suggested by Dr Bourke, would require 1 kg of peramine which is not only unfeasible but also unnecessary given that mice were dosed at a dose rate 2000 times greater than the maximum dose rate which could be ingested by a horse grazing herbage. Lambs dosed orally at a rate of 40 mg peramine/head/day for 5.5 days and at 80 mg peramine/head/day for a further 1.5 days showed no toxicity symptoms (Pownall et al. 1995).

Toxicity of chanoclavine

Our position on chanoclavine is that our results and those of others raise no concerns for farm animals and that there is no evidence that chanoclavine will cause a food safety issue. We did not

state that chanoclavine "must not be toxic" and acknowledge that further toxicity testing is required. This work will be conducted in rats following OECD guidelines.

Toxicity of terpendole E

We stand by our position that terpendole E is non-tremorgenic. Finch (unpublished data) tested terpendole E for tremorgenicity using a mouse bioassay and it was non-tremorgenic at 8 mg/kg. To put that into context lolitrem B gives significant tremors at 1 mg/kg so it is at the very least much less tremorgenic. From the structure of terpendole E it has no OH group in the 13 position so it is expected that this compound would not be capable of producing tremors at any dose rate. Furthermore, at this dose rate of 8 mg/kg terpendole E induced no adverse effects or any other signs of toxicity.

Rodents as test animals for mammalian toxicity

Despite Dr Bourke's view that rodents are "notoriously tolerant of toxins" the toxicology research community has adopted the rat or mouse as the preferred species for oral and inhalation testing, and for dermal testing, the rat or rabbit (United Nations 2007 – Part3 Health Hazards - http://www.unece.org/trans/danger/search?q=Toxicology+testing; See also http://www.alttox.org/ttrc/toxicity-tests/acute/).

Initial toxicity studies are routinely done in rodents which are considered an excellent model in the assessment of food contaminants and additives. Internationally recognised OECD testing guidelines 402, 403, 420, 423 and 425 all indicate that the preferred rodent species is the rat, although other rodent species may be used – these guidelines are referenced in OECD 1987, 2002a & b, 2008, and 2009. Oral administration is most commonly used for acute systemic toxicity testing – this is what we have used in our testing to date.

The principles of risk assessment are described in the review of Munday and Reeve (2013). This review also describes how the OECD guidelines used in our toxicity studies have been applied to the risk assessment of shellfish toxins. The results of these studies, using rodents, have been used to set regulatory limits of toxins in shellfish for human consumption.

Validity of methodology for toxicity testing

Dr Bourke mentions that for the toxicology work we have done to date that we have not specified the number of mice used. However, we have given the OECD guideline that we have used which specifies the number of live/death reversals or limit dose numbers. We closely followed accepted and documented international practice.

Horse oedema

In the case of equine fescue oedema which first occurred in Australia, this was a new form of toxicity to horses that had never been encountered anywhere before. Once identified we and our licensee company were able to quickly remove supply of this endophytic product from the market – this was done in 2010 and not 2013 as indicated in the submission by Dr Bourke. This ease of removal from the market was expedited because the endophyte is only found in the plant/seed and so by removal of the seed supply the product was terminated.

We disagree with Dr Bourke's view that the toxicity observed in horses is most likely due to the high levels of N-acetyl norloline (NANL) present in this endophytic product. Based on the figure of 2000 mg/kg N-acetyl norloline given in his submission, this would equate to a dose rate of 60 mg/kg in herbage which induces high toxicity to horses after 3 to 5 days grazing. In contrast, our toxicity testing of N-acetyl norloline in mice has showed not even a flicker of toxicity at the limit dose rate of 2000 mg/kg. While we disagree with Dr Bourke's statement that "rodents are notoriously tolerant of toxins" we acknowledge that a species difference is possible. However, given the much higher dose rate given to mice we consider that a species difference is unlikely to account for this non-toxic response in mice and that it is more likely that another causative agent is responsible for equine fescue oedema.

Horses have been tested (L Fletcher unpublished) on meadow fescue, and Mediterranean fescue cultivars with endophyte for effects of NANL concentrations. Oedema only occurred on the Mediterranean fescue cultivars with endophyte, but never on meadow fescue. At the time of horse grazing the average NANL level in Mediterranean fescue leaf was 1141 ppm compared to the NANL level in meadow fescue leaf of 699 ppm (61% of the Mediterranean leaf concentration). We would contend that it is unlikely that NANL is the causative agent of equine fescue oedema as it would have to be an incredibly steep dose response for horses to get very sick on the NANL concentrations shown for Mediterranean fescue but have no effect at all at half of that concentration as occurred with meadow fescue. It is improbable that such a steep dose response exists and more likely that equine fescue oedema is due to another unknown toxic factor and not NANL. Similarly for total loline concentration in leaf tissue (levels were 1140 ppm in Mediterranean fescue and 2676ppm for meadow fescue) it is unlikely that other loline compounds were the causal factor for equine fescue oedema.

Dr Bourke suggests that toxicity for horses occurs at a NANL oral dose of between 180 and 300 mg/kg live weight (being 60 mg/kg live weight per day). We would contend that if horses do get very sick on 60 mg/kg NANL but mice were completely unaffected by 2000 mg/kg that even with this species difference the evidence for NANL being responsible for equine fescue oedema is weak.

Managing endophytic seed/grain

We agree that selected endophytes can interact with their plant host in unpredictable ways and we have managed that effectively over the last 20 years with release of novel endophytes for grasses. At the first sign of any issue the endophyte strain can be withdrawn because it is managed though the host plant – it is not a free-living organism in nature. Withdraw the grass or cereal and the endophyte is nullified. This is easier to achieve with cereals because they have an annual sowing cycle.

Conclusion

We do agree with Dr Bourke that it is in everyone's interest to undertake extensive toxicity studies before the product is commercialised. It has always been our intention to do that. We already do this with our endophytic grass products which are tested at various stages of their development using rats, sheep, cattle and horses. Release from containment of these endophytes will allow sufficiently large areas to be grown to produce sufficient grain for the prescribed animal safety tests.

As clearly indicated in our application we will be doing appropriate animal testing before any commercial release of an endophytic grain product. If EPA wishes to make this release conditional on this animal testing occurring then we will accept this option.

References

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