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Devitalisation of feed grain by fumigation

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Executive summary

FLOT.124 aimed to address what were perceived to be three major biosecurity issues associated with imported feed grain by:

- Demonstrating the ability of EDN to devitalise four target grain commodities (maize, wheat, barley and sorghum). This was important as devitalising grain breaks the life cycle of any obligate pathogens (e.g. many viruses and pathogens).
- Devitalising contaminant weed seeds that might be potentially associated with imported grain; and
- Using surrogate pathogens to demonstrate a capability to devitalise pathogens that might be associated with imported grain.

The results demonstrated “in principle” feasibility of treating maize, barley, wheat and sorghum imported from the UK and USA along with some, but not all, exotic seeds tested. In addition, the efficacy of EDN was demonstrated against a selection of surrogate pathogens chosen to represent the pathogens deemed to be quarantine risks associated with the four commodities from the UK and USA. Based on these results a further project FLOT.127 was planned to extend the results of FLOT.124 and to demonstrate a commercial protocol using EDN.

FLOT.127 objectives were to:

- Extend the laboratory scale work towards large scale commodity fumigation,
- Confirm efficacy of EDN against insects,
- Assess control of quarantine weeds and surrogate weeds against doses up to the maximum treatment dose, and
- Verify efficacy against target pathogens of quarantine concern.

Results

Insects

Toxicity testing of insects included all stages of the Lesser Grain Borer (*Rhyzopertha dominica*), psocids (*Liposcelis entomophila.*) and the diapausing larvae of the Warehouse Beetle (*Trogoderma variabile*). There was no survival in any treatment. Treatments were approximately 10% the maximum target dose. Table 1 shows the results of these tests.

50kg trials

By applying concentrations below the flammability limit over five days, dosages up to 13800 mg h L^{-1} target were applied. This demonstrated that application of large doses of EDN to wheat, barley and maize up to the recommended maximum dose from FLOT.124 were feasible. All samples of maize taken from the treatments for germination were devitalised.

One tonne trials

One 500kg trial, to provide treated and un-treated maize for a weaner feeder trial, and four one tonne trials on maize were designed to demonstrate the preferred application technique

for commercial scale fumigation. A target dose of about 13800 mg h L^{-1} was applied by matching the addition of gas into a recirculated airflow with the loss and breakdown of EDN during the fumigation. Gas distribution was assessed by measured concentrations at several locations in the bin and samples of maize drawn from the bins for germination testing were killed in the first two trials. In the third trial were the grain was fumigated during winter, there was some survival of treated maize samples taken near the edge of the bin. This may have resulted either from uneven gas distribution in the bin or more likely very cold grain at the edges, below 5ºC, as EDN is less effective at low temperatures. In two further one tonne trials, where the grain was maintained above 20ºC during the fumigation, all maize samples tested for fumigation were killed. These trials also confirmed earlier estimates of the quantity of gas that would be required to treat bulks. Particularly, that about 3 kg/tonne would be required for maize, the likely import candidate, to reach the maximum target dose.

Weed seed testing

Though considerable effort was put into extending the list of weeds devitalised by EDN at doses up to 13800 mg h/L the essential result of FLOT.124 stands. Not all weed seeds are killed and other species remain untested because untreated control weed seeds failed to germinate. From limited trials at very high doses it would seem that hard coated weed seeds are able to effectively exclude EDN from penetrating the germ no matter how high the dose. Efforts to break this down with pre-treatment, including wetting to induce swelling, microwave treatment and ultrasound to disrupt the seed coat on some uncontrolled species were not successful. Given these results the only alternative is to exclude weed seeds from imported maize by a combination of sourcing from weed free production areas and or cleaning to reduce the residual risk to a level acceptable to Biosecurity Australia. This is feasible “in principle” though would add to the cost of sourcing grain for importation. However, if weeds are excluded from imported maize a reduced dose is possible making the cost of EDN treatment lower to offset these increased costs.

Grain sorting

Given the result of weed seed tests grain sorting as an alternative method to exclude weed seeds associated with maize from USA was evaluated from the literature. Removing weed seeds to a high level from imported shipment of maize is feasible and forms a major part of the import protocol for the importation of maize seed for the sweet corn industry. Three grain sorting and cleaning methods show promise and will need to be evaluated to determine the most cost effective and efficient. These methods are based on size, aspiration, and density in a fluidised bed or a combination of these.

Pathogens

The assessment of efficacy against target pathogens of concern included *Tilletia indica* (Karnal bunt), *Peronosclerospora sorghi* (sorghum downy mildew), *Tilletia controversa* (dwarf bunt) and *Ustilago maydis* (boil smut) and was conducted in collaboration with the USDA ARS.

EDN was tested on: 1.) naked spores; 2.) bunted seed, when this is a propagule in the life cycle of the pathogen; and 3.) spores dusted on maize. It was applied at120 mgL^{-1} over a period ranging from a few minutes to 5 days at 5, 17 and 22°C. Where appropriate, spores of treated material and untreated controls were plated out for assessment of efficacy. In the case of sorghum downy mildew, seed of a susceptible variety of sorghum was planted in soil inoculated with treatment and control spores as a bioassay of efficacy.

A pathogen associated with UK wheat, *Hymenula cerealis* (Cephalosporium Stripe) and sorghum smuts *Sporisorium sorghi* (Covered Kernel smut) and *Sporosorium cruentum*

(Loose Kernel Smut) were not available for testing. *Sporosorium relianum* was included as a surrogate for the sorghum smuts but test material was non-viable.

The naked teliospores of the three smut fungi (*T. indica, T. controversa* and *U. maydis)* were controlled and were more easily controlled than spores still contained within the fungal structure (sorus) with those spores that were dusted onto maize seed the most difficult to control.

Oospores of *S. sorghi* germinate poorly, if at all, on artificial medium. Treated oospores where mixed into the upper 5 cm layer of soil in a 2 X 2 inch plastic pot and the pots planted with seeds of a highly susceptible sorghum cultivar and placed in a growth chamber for disease development. Trace infection was observed in the untreated controls, and in one replication of the 1 hr treatment at 17°C. Cross-contamination of the treated oospores cannot be ruled out and though this result would indicate control the treatment may need to be repeated for confirmation.

Overall results, however, indicate that pathogens are relatively easy to control at the likely treatment schedule though a repeat trial for *P. sorghi* for confirmation was planned by our USDA collaborators. This is yet to be negotiated.

Commercial trials

Negotiations by BOC Ltd for the production of commercial quantities of EDN are complete and sufficient quantities of gas should be available for larger commercial trials once an agreement between BOC and MLA is concluded. EDN is currently in the assessment phase with APVMA for registration by BOC Australia and is now registered for use on timber. Whether Biosecurity Australia would require further proving trials beyond those reported here is not known but commercial trials of the application method will be required to ensure even distribution and efficacy in devitalisation of the imported commodity at the accepted recommended dose.

Recommendation

The use of EDN to fumigate imported maize to cover many but not all quarantine concerns is feasible. The remaining outstanding issue is control of associated weed seeds. Not all weed seeds are devitalised and a number remain untested because of availability or failure of untreated controls to germinate. Hence a protocol similar to that set out in the importation of Sweetcorn seed from Idaho, USA for sourcing and cleaning the shipment of maize prior to importation may be acceptable to Biosecurity Australia as the first step in a fumigation based protocol. In addition the shipment would be essentially free of insects and pathogens. It is then recommended that a single treatment with EDN at a quarantine facility at point of importation designed to cover the remaining quarantine issues by devitalising the commodity and killing any pathogens or insects, associated with the delivery pipeline, that may be picked up as a contaminant of grain handling pathways through which the shipment would need to pass as it is loaded onto ship.

As weed seeds will be excluded from the shipment by physical means it is recommended that the dose is set at approximately 6000 mg h/L EDN to control the remaining risks. This would be sufficient to devitalise the maize and control any contaminant pathogens picked up during handling and transport. As this is less than half the dose applied in the one tonne trials, it would also significantly reduce the cost of EDN applied, reduce the fumigation time and increase the logistic capacity of treating large bulks. It would also provide additional options for application e.g. periodic rather than continuous application of EDN.

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1. Background

Drought has a large impact on feed grain prices. Both graziers and feedlot operators are forced to pay higher prices for feed grain in efforts to sustain herds and production, and to maintain export contracts. Climate predictions increasingly indicate that such events may occur with greater frequency due to the atmosphere alteration associated with fossil fuel and other man-made emissions.

Previous attempts to import feed grain to alleviate high prices accompanying periods of local grain and feed shortage have been subjected to onerous protocols. One treatment adopted is hammer milling of imported feed grain to effectively devitalise the grain and any potential weeds, followed by steam treatment to remove pathogens. However, grain treated in this way is difficult to handle in feedlot operations. Grain has also been imported under quality specifications and then allowed only limited movement within metropolitan areas for chicken production. The only registered fumigant considered a candidate to effectively treat imported grain is methyl bromide, which is being phased out for uses other than quarantine and pre-shipment because it is an ozone depleting gas. Moreover, studies by Cassells *et al.* (1995) show that only partial devitalisation of maize and sorghum is achieved with methyl bromide, and they concluded that CT products (concentration × time) in excess of 17,000 mg h L^{-1} would be necessary to effectively treat the grain.

CSIRO has developed a new fumigant, ethanedinitrile (EDN), with the potential to treat imported grain. CSIRO was approached by the Australian grain industry to provide a proof of concept for an “Emergency Import Permit” during the 2002 drought. This interest subsequently developed into the FLOT.124 investigation which was continued under FLOT.127 the results of which are reported here.

FLOT.124

The objectives of FLOT.124, originally part of a larger proposal formulated to develop an import protocol for feed grain, were designed to provide the initial evaluation of the EDN concept, enabling Meat & Livestock Australia (MLA) to assess the merits of full commercialisation of EDN fumigation of grain. The objectives thus described laboratory scale experiments and aimed to address what were perceived to be the three major biosecurity issues associated with imported feed grain to:

- demonstrate the ability of EDN to kill the four target grain commodities (maize, wheat, barley and sorghum). This was important as devitalising grain breaks the life cycle of any obligate pathogens (e.g. many viruses and pathogens).
- devitalise contaminant weed seeds that might be potentially associated with imported grain; and
- demonstrate a capability to devitalise pathogens that might be associated with imported grain.

Other aspects, such as compatibility of materials and the behavior of the gas, were also included to discount possible barriers to application, and to formulate a dose and protocol for treatment on a larger scale.

The results of FLOT.124 demonstrated the “in principle” feasibility of treating maize, barley, wheat and sorghum imported from the UK and USA along with some, but not all, exotic seeds associated with these products. In addition, the efficacy of EDN was demonstrated

against a selection of surrogate pathogens chosen to represent the pathogens deemed to be quarantine risks associated with the four commodities from the UK and USA.

The commodities were relatively easy to devitalise with all those tested at 14% moisture content being devitalised at doses less than the discriminating dose of 2400 mg hL^{-1} used on weed seeds. The surrogate pathogens and weed seeds tested were more difficult to devitalise. A notional dose of 13800 mg hL^{-1} was set as a maximum dose based on the surrogate pathogen results. While assessing the efficacy of EDN, headspace gas loss, a measure of breakdown of the fumigant on the commodity, was found to differ markedly between commodities. This has implications on the final cost of treatment for each commodity; other target organisms, admixed with a commodity with higher headspace gas loss will be exposed to a lower dose than if they were mixed with a commodity with lower headspace gas losses. On this basis it was concluded that maize would be the most feasible commodity to treat with EDN, followed by wheat and then barley. Given the easy availability of the other commodities, and the variable feed value of sorghum, the management committee did not feel that work on sorghum beyond FLOT.124 would be justified.

FLOT.127

The objectives of FLOT.127 were designed to evaluate the feasibility of, and define treatment protocols for, the devitalisation of grain and possible contaminants of imported feed grain (maize, barley and wheat) using EDN.

This project aimed to extend the weed and pathogen work beyond the target species tested in FLOT.124, with particular emphasis on establishing whether the maximum dosage of EDN set (13800 mg h/L) was sufficient to devitalise the weed seeds and actual pathogens of quarantine concern, rather than the surrogates tested in the previous work.

Using a combination of laboratory work and staged fumigations of progressively larger quantities of commodity, the project aimed to define potential treatment protocols using EDN and investigate the treatment of maize, barley and wheat, and possible contaminants of imported feed grain. Several commercial scale treatments of maize would be scheduled once commercial quantities of EDN are available. These trials will provide a final data set on a protocol for EDN devitalisation of maize for submission to Biosecurity Australia.

The objectives of FLOT.127 are to:

1) Evaluate the feasibility of devitalisation of grain and specified insect, weed seed and pathogen contaminants of imported feed grain (maize, barley and wheat) using EDN

2) Define the most cost-effective treatment protocols for devitalisation of grain and specified insect, weed seed and pathogen contaminants of imported feed grain (maize, barley and wheat) using EDN and draft these in a format suitable for submission to Biosecurity Australia to initiate an import risk assessment.

3) Demonstrate the effectiveness of the EDN protocol to devitalise commercial scale quantities of maize (or another grain nominated by MLA) and specified insect, weed seed and pathogen contaminants.

Research Methods

The project consisted of two parts, laboratory work and varying scale test fumigations. Laboratory work was conducted to extend the work beyond the target contaminant species tested in project FLOT.124. An important component of this work was to verify the results obtained with surrogate pathogens by conducting work on the actual pathogens either in

Australia in the secure facility at CSIRO Geelong, or overseas if this was not possible. As permission to import pathogens into AAHL at Geelong was found to be too difficult, and securing suitable expertise in Australia not feasible, the work was conducted overseas. Tests on available pathogens were conducted in the USA at Fort Detrick, Maryland in collaboration with the USDA.

Additional supplies of weed seeds were sourced and screened in quarantine at the CSIRO Entomology, Canberra to identify which species can be controlled and at what dose. Grain storage insects were also screened. The results obtained from the laboratory work were used to refine the dose schedule developed as part of FLOT.124. This information was then used as a basis for the second part of the experimental work, which consisted of small, medium and large scale commodity fumigation. Small scale fumigations were conducted on three commodities (maize, barley and wheat). These were followed with medium (1 tonne) fumigations of maize and large scale (200-500 tonne) fumigations of maize were planned when sufficient quantities of EDN were made available by BOC, who are licensed to register and commercialise EDN. One of the other two commodities (barley or wheat) may be substituted for maize by MLA should this be deemed the most promising commodity for commercial application.

The purpose of the fumigation studies was to demonstrate efficacy of, and to develop the most efficient application of, EDN treatment of the chosen commodity. In each of these trials the spatial and temporal behaviour of the chemical was monitored and devitalisation of the commodity determined. This report summarises the results of experimental work and recommends a treatment regime for maize as well as providing recommendations for wheat and barley, specifying for the purposes of a quarantine import risk assessment which weeds, insects and pathogens are controlled and what exclusions are required.

EDN concentration was monitored during each application over the fumigation period to determine the dosage that would be received by any weeds or pathogens within the bulk commodity. Gas purity and concentrations were determined by gas chromatography. All treated material was equilibrated to a relative humidity appropriate to that of imported grain. Test organisms included grain storage pests (Activity 1), weeds (Activity 2) and pathogens (Activity 3).

2. Toxicity testing of insects

The tolerance of insects to ethandinitrile was assessed by (Hooper *et al.*, 2003. Toxicity of cyanogen to insects of stored grain. *Pest Management science* **59**: 353-357.). The purpose of this activity was to provide additional data for all life stages of the Lesser Grain Borer (*Rhyzopertha dominica*) and psocids (*Liposcelis spp.*), as well as diapausing larvae of the Warehouse Beetle (*Trogoderma variabile*). These were tested at a single dose in order to demonstrate the efficacy of the gas against the various life stages and particularly against the resilient diapausing stage of *Trogoderma*.

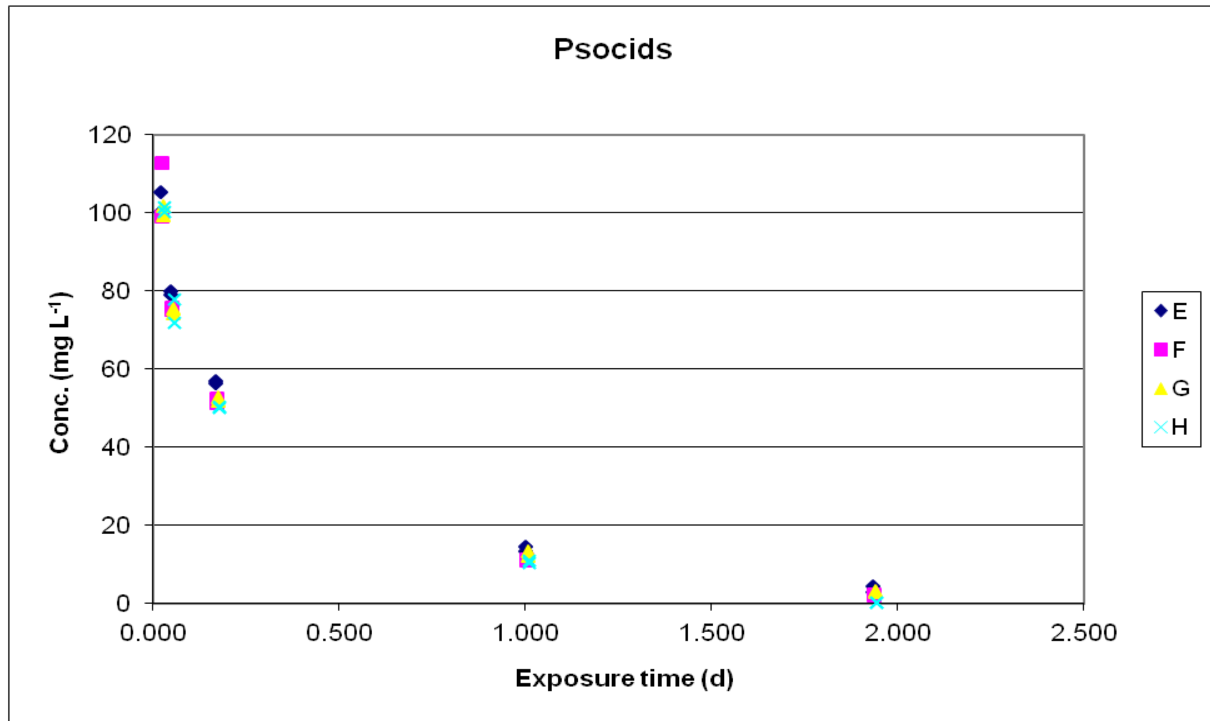
Methods

Toxicity testing of insects included the grain storage pests Warehouse Beetle (*Trogoderma variabile*), Lesser Grain Borer (*Rhyzopertha dominica* F.), chosen as surrogates of Khapra Beetle (*Trogoderma granarium* Everts) and the Larger Grain Borer (*Prostephanus truncatus* Horn), and psocids (*Liposcelis spp.*). The work aim was to establish that EDN dosage regimes applied for devitalisation are sufficient to control the diapausing larvae of Warehouse Beetle (*Trogoderma variabile*), all stages of Lesser Grain Borer (*Rhyzopertha dominica*) and psocids (*Liposcelis spp.*). Culture medium containing all stages or diapusing

larvae of *T. variabile* was placed into desiccators conditioned overnight to 75% RH. An Initial dose of 120 mg/L EDN was applied and decay monitored over two days. Dosage was calculated from area under the curve to provide an estimate of the dosage received by the insects. Un-dosed controls were treated the same in every other respect. Treated media containing insects was recovered and emergence measured over 8 weeks to assess mortality and estimate control numbers in whole cultures.

Results and discussion

The initial dose of 100 mg/L EDN was sorbed after two days. Figure 1 shows a typical headspace concentration for the psocid exposure.



Actual concentration by time (Cxt) dosage was estimated from the area under the curve and these are reported for each replicate in Table 1.

Table 1 shows estimates of numbers treated from control emergence. There was no survival in any samples treated with EDN. Given that the doses received shown in column four are well below the treatment dose for devitalising the selected commodity probability of survival of insect pests in commercial treatments would be very low.

Table 1. Toxicity of EDN to all stages of the Lesser Grain Borer (Rhyzopertha dominica), psocids (Liposcelis spp.) and the diapausing larvae of the Warehouse Beetle (Trogoderma variabile).

Replicate	Species	Dose	cxt mg h/L	Survival	Total survival
A	*Rhyzopertha dominica* (all stages)	Cont	0	3752	15647
B			0	4189	
C			0	4099	
D			0	3607	
A		100 mg/L	847	0	0
B			810	0	
C			773	0	
D			810	0	
A	*Liposcelis entomophila* (all stages *)*	Cont	0	>9223	>37187
B			0	>9379	
C			0	>9184	
D			0	>9401	
A		100 mg/L	1025	0	0
B			887	0	
C			960	0	
D			792	0	
A	*Trogoderma variable* (diapusing larvae)	Cont	0	188	1316
B			0	185	
C			0	188	
D			0	187	
E			0	189	
F			0	189	
G			0	190	
A		100 mg/L	555	0	0
B			911	0	
C			697	0	
D			567	0	
E			945	0	
F			781	0	
G			779	0	
H			618	0	

3. EDN tolerance of testable weeds

Background

A list of weeds of quarantine concern in maize from the USA was compiled by Biosecurity Australia as part of the Maize IRA. This list also essentially covered possible weeds of wheat from the USA. As wheat is grown in rotation with maize, the weeds associated with wheat are considered possible contaminants of the crop and the supply chain. This list is contained within the Maize IRA at the Australian Forestry Fisheries and Agriculture website at:

http://www.affa.gov.au/corporate_docs/publications/pdf/market_access/biosecurity/plant/final_maize.pdf

However no publicly available list of potential weed species associated with UK wheat (and barley) was available, so potential weeds associated with these commodities was compiled from literature sources by David Rees, CSIRO Entomology. The completed list included plant species recorded as weeds of wheat crops in the UK, including plant inhabitants of the field margins of wheat crops. The list includes:

- Weeds of potential economic importance to Australia and not yet present in Australia, or present but not widely distributed and being officially controlled.
- Weeds listed as ‘noxious’ by any state or territory legislation which would thus be of quarantine concern if present in imported grain.
- No attempt was made to assess the difference in herbicide tolerance between strains of weed species that are found in both the UK and Australia, nor to determine whether they have been assessed for weediness and cleared for import by Biosecurity Australia using their ‘weed risk assessment’ process.

Biosecurity Australia was furnished with the results of the study in the January 2003 and this body of work is attached as Appendix B of the FLOT.124 report.

Weed testing in FLOT.124

As research on quarantine weeds is subject to both regulation and availability, not all quarantine weeds could be tested. Those for which both permit and seed have been obtained were tested as part of FLOT.124. Surrogate species for weed species which were unavailable were also subjected to testing. The testing consisted of a “discriminating dose” (261 mg L^{-1} for 5 days at 2.5% fill ratio and seed equilibrated to 70% RH; dose equivalent to 2358 mg h L^{-1}) which was devised to identify the most tolerant seed for further study or for categorisation as “not controlled by fumigation” as part of a future fumigation protocol. In the course of this test a considerable portion was deemed to be not testable due to the lack of adequate germination in the control (untreated seed). This may have been due to lack of seed viability or alternatively ineffective germination methodology. Thus there is a large number of seed which were either not testable or which were unavailable. Activity 2 is designed to address this to the greatest extent possible. This will be achieved in the first instance by attempting to source as much of this seed as possible for testing, and failing that by sourcing surrogates where non have been tested to date and finally by revising germination protocols where the seed were categorised as not testable in the initial discriminating dose applied in FLOT.124.

Weed quarantine permit

The original permit for quarantine work on weeds covered the initial import of weed seeds and a second import which was made late in the FLOT.124 project to supply additional seeds for anticipated needs. This permit specified the Black Mountain Quarantine Facility as the Quarantine Approved Premises and stipulated the protocols to be observed for the fumigation work. In the time between end of FLOT.124 and start of FLOT.127 the permit lapsed. While the seed is still being stored in the quarantine facility no further work will be undertaken until the matter of a permit has been finalised. This is being organised by David Rees who compiled the original weed list and arranged the original permit. The new acquisitions of seed will also have to wait until a new permit can be organised. Should the permit not be approved the existing seed will have to be destroyed in compliance with the original permit.

Weeds to be tested

The availability of weed seed has been reviewed by Joel Armstrong. He has compiled a list of weeds and surrogates which can be obtained from Herbiseed (New Farm, Mire Lane, West End, Twyford, RG10 0NJ, England. Tel: +44(0)1189349464 Fax: +44(0)1189241996 Email: sales@herbiseed.com). The current status of the weeds is summarised in the tables below. In addition Australian researchers who work on weeds were canvassed in an attempt to acquire weeds which may be of quarantine concern, but which already occur in Australia. Michael Moerkerk of DPI Victoria (Michael.moerkerk@dpi.vic.gov.au) was the only researcher identified with a relevant collection of weed seeds. He is currently reviewing our list of weeds to determine if he can supply some of the species. The following is a list of seed which is still to be tested. Many have already had surrogate species tested. Some are additional surrogates which will be tested if available, and a few are highlighted as seed for which no source has yet been found, and which no surrogate exists. It is anticipated that this seed will be tested at the discriminating dose in batches as it becomes available. Further effort will be made to acquire additional still outstanding seed during the life of the project.

1) The weeds in this table were all tested in FLOT.124. However, the controls did not successfully germinate, and an alternative germination methodology is required to overcome this problem.

Weed species we do have in Quarantine + quantities

SAN	Family	*Genus*	*Species*	Amount
10127	Asclepiadaceae	*Asclepias*	*syriaca*	100g
10129	Asteraceae	*Ambrosia*	*trifida*	500g
10132	Asteraceae	*Cirsium*	*arvense*	20g
10133	Asteraceae	*Cirsium*	*vulgare*	20g
10136	Asteraceae	*Senecio*	*jacobaea*	10g
10153	Lamiaceae	*Galeopsis*	*tetrahit*	25g
10157	Lamiaceae	*Stachys*	*sylvatica*	10g
10163	Poaceae	*Cenchrus*	*incertus*	200g
10162	Poaceae	*Cenchrus*	*longispinus*	200g
10169	Poaceae	*Panicum*	*fasciculatum*	5g
10179	Rubiaceae	*Galium*	*aparine*	100g
10180	Solanaceae	*Datura*	*stromonium*	50g
10124	Umbelliferae	*Anthriscus*	*sylvestris*	50g
10125	Umbelliferae	*Heracleum*	*sphondylium*	60g

2) This list is species which could not be sourced but which a surrogate exists. These surrogates in normal text were successfully tested in FLOT.124 and require no further testing unless a new seed source is identified. Surrogates which are in bold font were not successfully germinated during FLOT.124 study. These will need modified germination protocols as above.

Weed species we have surrogates for in Quarantine.

Family	*Genus*	*species*	SAN number	Surrogate	Amount
Amaranthanceae	*Amaranthus*	*hybridus* (herbicide resistant)	10121	*A. chlorostachys*	10g
Amaranthanceae	*Amaranthus*	*rudis* (triazine resistant)	10122	*A. palmeri*	10g
Amaranthanceae	*Amaranthus*	*tamariscinus*	10123	*A. retroflexus*	10g
Asteraceae	*Ambrosia*	*grayi*	10128	*A. artemisiifolia*	60g
Asteraceae	*Bidens*	*aurea*	10130	***B.tripartia***	25g
Asteraceae	*Erigeron*	*annuus*	10134	***E. candensis***	15g
Chenopodiaceae	*Salsola*	*collina*	10145	*S. kali*	15g
Convolvulaceae	*Ipomoea*	*turbinate*	10147	*I. hederacea, I. lagunosa, I. purpurea*	200g of each species
Euphorbiaceae	*Chamaesyce (Euphorbia)*	*maculata (supina)*	10152	***E. lathyris***	200g
Lamiaceae	*Lamium*	*album*	10154	***L. purpureum***	10g
Poaceae	*Urochloa (syn Brachiaria)*	*platyphylla*	10175	***U. panicoides***	25g
Solanaceae	*Solanum*	*ptycanthum*	10181	***S. nigrum***	15g
Umbelliferae	*Torilis*	*japonica*	10126	*T. arvensis*	10g

3) These species have surrogates which are taken from locally available seed sources. These have been transferred to the quarantine facility where the fumigation/germination studies will be undertaken.

Weed species we have surrogates for in Coolroom

SAN	Family	*Genus*	*species*	SAN (surrogate)	Locally available surrogate
	Amaranthanceae	*Amaranthus*	*hybridus* (herbicide resistant)	10016	A. hypochondriacus
	Asteraceae	*Acanthospermum*	*hispidum*	10013	Arctium lappa
	Asteraceae	*Bidens*	*aurea*	10044	Lactuca sativa
	Asteraceae	*Erigeron*	*annuus*	10045	Echinacea purpurea
	Asteraceae	*Eupatorium*	*capillifolium*	10038	Centaurea cyanus
10136	Asteraceae	*Senecio*	*jacobaea*	10052	Tanacetum vulgare
	Asteraceae	*Verbesina*	*encelioides*	10039	Taraxacum officinale
	Euphorbiaceae	*Euphorbia*	*platyphyllos*	10050	E. marginata
	Lamiaceae	*Galeopsis*	*angustifolia*	10042	Hyssopus officinalis
	Lamiaceae	*Glechoma*	*hederacea*	10049	Scutellaria baicalensis
	Polygonaceae	*Brunnichia*	*ovata*	10098/10021	Fagopyrum esculentum
	Scrophulariaceae	*Striga*	*asiatica*	10041	Digitalis purpurea
10124	Umbelliferae	*Anthriscus*	*sylvestris*	10036	A. cerefolium
10125	Umbelliferae	*Heracleum*	*sphondylium*	10037	Coriandum sativum

4) These seed currently have no identified supplier and no surrogate has been found.

Seeds we cannot get as at Aug 2005

Apocynaceae	*Apocynum*	*cannabinum*
Asclepiadaceae	*Cyanachum (Ampelamus)*	*laeve*
Equisetaceae	*Equisetum*	*arvense*
Fabaceae	*Aeschynomene*	*virginica*
Fabaceae	*Senna*	*obtusifolia*
Menispermaceae	*Cocculus*	*carolinus*
Poaceae	*Muhlenbergia*	*frondosa*

5) This is a list of seed which is to be ordered from Herbiseed on the approval of AQIS quarantine permit.

Weed species to order from Herbiseed

Family	*Genus*	*species*	Rate		Cost **(£)**	Amount (g)
Asteraceae	*Acanthospermum*	*hispidum*	100g @ £50		50	100
Asteraceae	*Eupatorium*	*capillifolium*	E. cannabinum 25g @ £26	surrogate	26	25
Asteraceae	*Helianthus*	*annuus (herbicide resistant)*	35g @ £7		7	35
Boraginaceae	*Myosotis*	*avensis*	35g @ £6		6	35
Brassicaceae	*Berteroa*	*incana*	Alyssum saxatile 5g @ £5	surrogate	5	5
Brassicaceae	*Conringia*	*orientalis*	5g @ £6		6	5
Cucurbitaceae	Sicyos	colocynthis	25g @ £17		17	25
Cyperaceae	*Cyperus*	*esculentus*	Cyperus iria 3g @ £10	surrogate	10	3
Cyperaceae	*Cyperus*	*rotundus*	5g @ £4		4	5
Poaceae	*Eriochloa*	*villosa*	25g @ £11		11	25
Poaceae	*Panicum*	*ramosum*	10168 P. dichotomiflorum 5g @ £5	surrogate	5	5
Poaceae	*Panicum*	*texanum*	10167 P. capillare 5g @ £4	surrogate	4	5
Poaceae	*Paspalum*	*boscianum*	5g @ £3		3	5
Poaceae	*Sorghum*	*halpepense*	25 & £7		7	25
Polygonaceae	*Polygonum*	lapathifolium	100g @ £8		8	100
Rosaceae	*Rubus*	*fruiticosus and relaitives*	10g @ £8		8	10
Solanaceae	*Physalis*	alkekengi	10g @ £8		8	10
	Avena	*strigosa*	35g @ £13		13	35

Fumigation method

The following methodology is that of the discriminating dose applied to weeds in FLOT.124. It was applied to all seed available for retesting as part of the above criteria. By adjusting fill ratio it was possible to achieve very similar CT products by applying a similar dose to that described in the experimental protocol below. In practice different seed will absorb gas at different rates so the CT product may vary considerably. By monitoring the sorption over the experimental timeframe it may be possible to either attenuate the experiment early or add more gas to achieve a CT product comparable to the discriminating dose of 2358 mg h L^{-1}.

The test seed was equilibrated at 70% RH and placed in steel micromesh containers so that the seed could not escape confinement. These containers were transferred to a gas tight pressure tested container, giving a fill ratio of approximately 2.5%. The drum was then sealed and transferred from the quarantine facility to the Entomology laboratories for fumigation (as per the quarantine import conditions) where a dose of 261 mg L^{-1} was applied as follows. EDN sourced from gas bottles was collected in a Tedlar bag and analysed to determine percent purity. This was undertaken using a Gas Density Balance, Tracor 220 M (Tracor Inc., Austin, TX, USA) with a 1 m × 5 mm glass column packed with Porapak Q 100/120 mesh (Alltech Associates: Deerfield, IL, USA) run at 105°C and a carrier gas (N_2) flow of 150 mL min^{-1} using 1,1,1,2 tetrafluoroethane as a reference gas. The quantity of gas needed to achieve target concentrations was calculated after correcting for pressure and temperature. Based on these calculations a quantity of gas was pumped into the container. Pressure balance was maintained by the collapse of an internal air bladder ported to the external atmosphere.

The container was then incubated at 25°C for a period of 5 d. During this time headspace concentration was measured by gas chromatography (GC) using a flame ionisation detector (FID) equipped Shimadzu GC6AM (Shimadzu Seisakusho, Kyoto, Japan). The column used was a 1 m × 3 mm glass column packed with HaysSep Q (Alltech Associates, Deerfield, IL, USA) run at 80°C and with a carrier gas (N_2) flow of 40 mL min^{-1}.

At the completion of the fumigation the drum was first aired, and then re-sealed prior to transfer back to the quarantine facility where germination assessments of the treated seed were compared to those of untreated control seed. Chemical or physical pre-treatments were applied to the seed as dictated by ISTA or published seed germination methods. Germinations were undertaken on wetted filter paper in 15 cm Petri dishes, or on rolled paper in plastic bags, and incubated under appropriate light and temperature conditions. In some cases alternative seed pre-treatments and surface sterilisation techniques were employed to increase germination. The germinated seed was assessed according to ISTA rules, and the positive germination results reflect the combined numbers of germinated, germinated low vigour, and germinated abnormal seed. A CT (Concentration × Time) product was calculated for the dose based on the headspace concentration measured over the duration of the experiment. The calculation was based on the least squares method.

In addition some weed seeds not controlled by EDN were subject to treatment by microwave, ultrasound or 1% extra moisture prior to fumigation to determine if these treatments were likely to make them more susceptible to devitalisation.

The seeds were subject to

- Microwave exposure at 30, 60, 90 seconds;
- Sprayed on moisture 1% with seeds in maize matrix;
- Ultrasound exposure at 30, 60, 90 seconds.

The seeds were then fumigated with EDN at the discriminating dosage and then assessed for germinability.

Results and discussion

As for FLOT.124, not all weed seeds were controlled by fumigation with EDN. Table 1 shows species controlled by EDN at the discriminating dosage or the maximum feasible dose. Table 2 shows those species that are partially controlled. Table 3 shows those weeds not controlled were control germinations were ok and Table 4 shows those weeds where failure of control germination means that these species remains untested. Table 5 lists unsourced species on the US list of weeds of quarantine interest.

One pertinent result shown in Table 2 is that some species shown as devitalised in FLOT.124 where not completely devitalised when fresh seed was tested. This further supports the probability that seed coat integrity of hard coated seeds can prevent EDN from reaching the germ.

Attempts to disrupt the seed coat with pre-treatment's of increased moisture, microwaves and with ultrasound were essentially unsuccessful in increasing efficacy of EDN with selected weeds. Even if successful these treatments would have been difficult to include in a timely treatment protocol.

Table 1. Weed species tested that were devitalised by EDN showing % germination tested / % germination in controls for a range of doses ($mg.hL^{-1}$)

		C2N2 Fumigation Dose: $mg.hL^{-1}$						
Species	**Surrogate of**	**Discriminating Dose 2.5K-3K**			**6K**	**9K**	**14K**	**20K**
*Ambrosia artemisiifolia**		0/97.3	0/0					
*Cyperus iria***		0/79.8	0.8/66.5		0/66.5			
*Cyperus rotundas***	*Galeopsis angustifolia*	1.5/93.5			0.8/83.7			
*Datura stramonium**		0/43	1/87.5		0/80			
*Echinochloa crus-galli 1**	*Eupatorium capillifolium*	0/75	1/71.5		0/60.3			
*Eriochola villosa***		0/73	0/57.3					
*Hyssopus officinalis**		0/57	0/32					
*Mentha arvensis**	*Ambrosia grayi*	0/28.3	0.2/6.3	1.3/12.8	0/5.8	0/6	0/7	0/5
*Panicum capillare**		0/32.3	0/56.3	0/25.8				
*Panicum dichotomiflorum**		x	0/92					0/16
*Polygonum pensylvanicum**		0/26.3	0/10.3					
*Salvia verbenaca**		0.3/62.3	0/65					0/64
*Setaria faberi**		0/26.	0/13.3					
*Setaria verticillata***		0/24.8	0/32.3		0/21.8	0/24.8	0/22.5	
*Xanthium spinosum***		0/50.3	0.8/49.8					

** US List ** Not on the US List*

Table 2. Weed seeds that were partially devitalised by EDN showing % germination tested / % germination in controls for a range of doses(mg.hL^{-1})

		C2N2 Fumigation Level: mg.hL^{-1}						
Species	**Surrogate of**	**Discriminating Dose 2.5K-3K**			**6K**	**9K**	**14K**	**20K**
*Acanthospermum hispidum**		0.3/75.8			76.9/99	80/99.5	83.6/97.3	
*Amaranthus chlorostachys**		0.3/27.8			8.5/39.8	11.5/44	7.3/49.3	
*Amaranthus palmeri**		9.5/31.6						
*Amaranthus retroflexus**		0/44.5	1.3/63.3	3.8/66.8	1.5/71			
*Avena strigosa***		0/70.8	7.5/34		38.5/89.5			
*Bidens tripartia***		0/13.5	5.9/71.8					
*Bromus tectorum***		0/55.8	2.8/6.8		5/11.3			
*Cenchrus longispinus**		0/63.2						
*Chenopodium album**		0.3/99.8			66/91.9	56.3/81		
*Chrysanthemum segetum***		75/85			4.3/44.8	9.8/44.5	4.4/36.8	
*Cirsium arvense***		0/39.3	8.8/66.8		11.8/76.8			
		22/77	41.5/67.3					
*Cirsium vulgare**	*Amaranthus hybridus*	55.3/66.3						45/59.3
*Convolvulus arvensis***		x	25.3/42.3		37.3/49.5	33/36.8	23/24.8	
*Cynodon dactylon**	*Lolium multiflorum*	0/85.5	45.5/68.3		30.8/68.8			
*Cyperus esculentus (seed)**		0/81.3	33/42.75		28.3/34.3			
*Cyperus esculentus (Tubers)**	*Amaranthus rudis*	31.5/49	54.3/97.3	16/32.7				43.8/99.5
*Fagopyrum esculentum**		93/95.3			39.3/67	39.5/67	40/51	
*Galium aparine**		6.5/24	2.8/22.5		3.5/24.3	0.8/19	3.3/26	
*Lolium perenne***		0/39	7/40.8	4/50.3	3.0/39	0.5/23.5	8.5/50.8	
*Myosotis arvensis***	*Torilis japonica*	0/14.8	48/68.3		22.5/33.5	19/32.5	43.8/61.3	
*Pennisetum glaucum***		0.5/19.8						
*Tanacetum vulgare**		x	21.5/48.5			15/29		0/3.8
*Thlaspi arvense**		15.5/38.8			2.3/37.5	3/43.8	1.8/31.3	
		6.3/37.8	6.5/25.3					
*Torilis arvensis**	*Amaranthus tamariscinus*	52.3/76.8			17.0/53.3	45.8/77.5	47.3/66	
*Xanthium strumarium**	*Brunnichia ovata*	5/66.5			4.5/60	10.3/63	17/80.5	

** US List ** Not on the US List*

Table 3 Weeds not devitalised by EDN showing % germination tested / % germination in controls for a range of doses(mg.hL^{-1})

Species	Surrogate of	C2N2 Fumigation Dose: mg.hL^{-1} Discriminating Dose 2.5K-3K		6K	9K	14K	20K
						37.8/52.5	
*Abutilon theophrasti**		38.3/54.3	99.5/98.8	37.8/52.5		18/23	18/16
*Apera spica-venti**	*Senna obtusifolia*	54.5/69		24.3/61.3			
*Brassica juncea***	*Senecio jacobaea*	6.3/4.3		1.5/2.3	3.8/3.3	4/3.8	
		x	5.2/0.2				
*Bromus commutatus**	*Bidens aurea*	3.3/8.8		0.3/2.8	0.3/6.5		6.5/4
*Conringia orientalis**	*Aeschynomene virginica*	78.3/94.3		70.3/96.8			
*Digitalis purpurea***		80.3/99.3	96.8/98.8	32.5/92.3		88.3/91.5	
*Galeopsis tetrahit**	*syn. Brassica japonica*	22/28		12.5/11.3	9.8/8.5	15/16.4	
*Helianthus annus**	*Ipomea turbinata*	98.5/99.3	99/99	99/99	98/97	98/99	
*Ipomoea hederacea**		0/93	91.8/90	90.3/93.8			
*Ipomoea lacunosa***		0/65	36.3/17.5	29/12.8			
*Ipomoea purpurea**		0/44.3	26.5/37.5	8/7.5	16.3/17	9.8/8	
*Jacquemontia tamnifolia**	*Ipomea turbinata*	10.5/60.3	90/96	96/94	97/94	94/93	
*Kochia scoparia***		0/88.5	58.5/61.3	54.8/64.3			
*Lablab (dolichos) purpurea**	*Solanum ptycanthum*	x	4.5/13	6.8/9.3	8.5/10	7.3/12	
*Matricaria perforata**		49.8/53		52/66.8	71.8/70	58.8/54.3	
*Phleum pratense**	*Striga asiatica*	30/60.8		52.5/47.8	47/49.3	54.8/58.3	
*Physalis alkekengi*i*		7.3/21		26/30.3	24.5/29	23.5/31	
*Polygonum aviculare***		0/54	17.3/24.5	11.5/19.5			
*Raphanus raphanistrum***		6.5/7.8		2.1/5.5	2.7/6.9	1.3/3.3	
		0/12.3	17.8/20.3				
*Senna artemesioides**		25/22		16.3/11.8	13/17.5	9/6.3	
*Sicyos colocynthis**		10.5/10.5					
*Solanum nigrum***		0/79	61.3/64.3	53.3/70.3			
*Sorghum halepense**	*syn. Bassia scoparia*	0/46.3	84.5/89.5	64/81.8			

** US List ** Not on the US List*

Table 4, Weeds that remain untested because of failure for controls to germinate

		C2N2 Fumigation Level: $mg.hL^{-1}$						
Species	**Surrogate of**	**Discriminating Dose 2.5K-3K**			**6K**	**9K**	**14K**	**20K**
*Asclepias syriaca**		0/0	0/0.3		0/0	0/0.3	0.3/0	
*Cirsium vulgare***		0/0			0/0			
*Polygonum lapathifolium**		x	1.3/3.3	0.3/3.3	0/2.3	0/1.75	0.3/2.3	0/4
*Ambrosia trifida***		0/3.8	0/2		0/2.8	0/4.5	0/3.25	
*Anthriscus sylvestris**		0/73.5	0/0	0/3.8	0/4.5	0/1	0/8.8	
*Apocynum cannabinum**		x	0/0	0/0				
*Arctium lappa***		0/0	0/0.3	0/0				
*Cirsium arvense***	*Acanthospermum hispidum*	0/0						
*Erigeron canadensis**		x	0/10.5	0.3/1.8				0/0
*Euphorbia lathyris**		x	0/0.3					
*Euphorbia marginata**	*Erigeron annuus*	x	0/0	0/0				0/0
*Galeopsis tetrahit**	*Chamaesyce maculata (supina)*	0/0.3	0/0	0/0				
*Helianthus annus***	*Euphorbia platyphyllos*							
*Heracleum sphondylium***		x	0/0.3	0/0				0/0
*Lamium purpureum**		0/0	0/0					
*Paspalum fasciculatum***		x	0/0	0/0				0/0
*Salsola kali***	*Lamium album*	x	0/0.5	0/0				0/0
*Scutellaria baicalensis***		x	0/0	0/0				
*Senecio jacobaea**		0/11	0/0	0/0				0/0
*Sicyos angulatus***	*Glechoma hederacea*	0/0	0/0					
*Stachys sylvatica***		0/0	0/0	0/0				0/0
*Taraxacum officinale**		0/0.3	0/0.5					
*Urochloa panicoides***		x	0/0	0/0				0/0
*Amaranthus arenicola**	*Verbesina encelioides*	0/0	0/0					
*Berteroa incarna**	*Urochloa platyphylla*	x	0/0.3					0/0

** US List ** Not on the US List*

Table 5. Weeds on the quarantine list that remain untested because suitable seed could not be sourced

Species	**Surrogate of**
Cocculus carolinus	see other Amaranthus results
Cynanchum laeve	
Equisetum arvense	
Muhlenbergia frondosa	
Panicum ramosum	
Panicum texanum	
Paspalum boscianum	
Physalis heterophylla	
Rubus allegheniensis	see other Paspalum result
Rubus fruiticosus	see other Physalis result
Salsola collina	
Senna (syn. Cassia) obtusifolia	
Datura inoxia	
Datura inoxia (resistant to ALS herbicides)	see result for Senna (syn. Cassia) artemisioides
Panicum fasciculatum var. reticulatum	see other Datura result
Polygonum bungeanum	see other Panicum results.
Salvia reflexa	see other Polygonum results
Setaria lutescens	see result for S. verbenaca
Sorghum x almum	see result for S. verticillata
Cenchrus longispinus	see other Sorghum result

4. Grain sorting

Given the result of weed seed tests, grain sorting as an alternative method to exclude weed seeds associated with maize from USA was evaluated from the literature. Removing weed seeds to a high level from imported shipment of maize is feasible and forms a major part of the import protocol for the importation of maize seed for the sweet corn industry. Three grain sorting and cleaning methods show promise and would need to be evaluated to determine the most cost effective and efficient. These methods are based on size, aspiration and density in a fluidised bed. Optical sorting is also an effective method of producing a clean sample. These methods in conjunction with careful sourcing of maize to reduce weed contamination may be an acceptable method of excluding weed seeds from imported maize.

References

U.S. Congress, Office of Technology Assessment, Enhancing the Quality *of U.S. Grain for International Trade,* OTA-F-399 (Washington, DC: U.S. Government Printing Office, February **1989).**

Final IRA paper: importation of sweetcorn seed (Zea mays L.) from Idaho (United States of America) for the purpose of field sowing in Australia

5. Verification of pathogen surrogates against actual incursion risks

A list of potential pathogens associated with the various commodities was supplied by Biosecurity Australia (Appendix 2). For the most promising commodity, maize from the US, the pathogen of interest is *Peronosclerospora sorghi* (sorghum downy mildew). The inclusion of wheat and barley from the UK would require additional work on the pathogens *Tilletia controversa* (dwarf bunt), *Ustilago tritici* (loose smut of wheat) and *Hymenula cerealis* (Cephalosporium Stripe). With the inclusion of wheat and barley sourced from the US, *Tilletia indica* (Karnal bunt) would need to be added to the list. It was assumed that by excluding sorghum from consideration it would be acceptable to Biosecurity Australia to exclude *Sporisorium sorghi* (Covered Kernel Smut) and *Sporosorium cruentum* (Loose Kernel Smut) from the testing regime. However, propagules for study were obtained by collaboration with pathologists in the US. The work was undertaken at Fort Detrick, Maryland with the USDA in the US. Using the results of surrogate studies as a guide, the project will test EDN on the corresponding pathogens listed in Appendix 2 as potential contaminants of concern in wheat and barley from the UK and maize, wheat and barley from the US. These results will be used to modify protocols if necessary. Small medium and large scale commodity fumigation investigations will be conducted to scale up the treatment protocol, culminating in a commercial scale verification trial.

This study reports results from assessments of efficacy against target pathogens of quarantine concern including *Tilletia indica* Mitra (Karnal Bunt), *Peronosclerospora sorghi* Weston & Uppal (Sorghum Downy Mildew), *Tilletia controversa* Kühn (Dwarf Bunt) and *Ustilago maydis* (DC.) Corda (Boil Smut), which is being conducted by the CSIRO in collaboration with the USDA ARS.

EDN is a colourless gas with an almond-like odour; its chemical and physical properties are listed in Table 1. EDN has been patented by the CSIRO (Desmarchelier and Ren 1996[6]) as a new fumigant effective against insects and micro-organisms. It has a threshold limit value (TLV) of 10 ppm, which compares favourably with 5 ppm for methyl bromide.

Materials and methods

The efficacy of EDN was tested on naked spores, bunted seed, when this is a propagule in the life cycle of the pathogen and spores dusted on maize. Three replicates of each were put into open Ependorf tubes and placed into open desiccators of measured volume, allowed to equilibrate to the 75% relative humidity overnight. The lids were then closed to seal the tubes, and injected with EDN through a gas septum port, having first withdrawn an equivalent volume of air to prevent desiccator lids from popping. In the case of P. sorghi, homogenised infected leaf material with oospores was placed into small Nitex® bags made of 20 µm pore-size polyester screen and placed into racks in the desiccator.

EDN was applied at 120 mgL^{-1} and held at 5, 17 and 22°C. Times of exposure were 10, 25, 60, 120, 250, 500, 1000, 1750, 3000, 4500, 7000 minutes. After treatment spores of treated material and untreated controls were plated out for assessment of efficacy. In the case of sorghum downy mildew, seed of a susceptible variety of sorghum was inoculated with treatment and control spores and planted out as a bioassay of efficacy.

The EDN was generated in the laboratory in a fume hood by slowly injecting saturated KCN into hot (95°C) $CuSO_4$. The air in an inverted bell, fitted with a gas sampling septum, was first withdrawn filling the bell with the hot $CuSO_4$ The generated gas was then transferred by syringe into a Tedlar® gas sampling bag and more EDN generated until sufficient for the days doses was made. After cooling to room temperature percent purity was analysed using a Thermal Conductivity Detector (TCD) fitted to an SRI model 8610C gas chromatograph using a 3 foot 1/8 inch column packed with Porapak Q 80/100 mesh, run at 100ºC with a carrier gas (He) 20 mL-1. Purity was measured from 78 to 89 % which reflected the temperature of the $CuSO_4$.

The quantity of EDN needed to achieve target concentrations in each desiccator was calculated based on percent purity from the TCD analysis and desiccator volume. Exposure concentrations were then measured by taking samples with a gastight syringe through a gas sampling septum and analysing them with a Flame Ionisation Detector (FID) using the same column and GC. Concentrations for the longer exposures were topped up from time to time to maintain the concentration as near to 120 mg/L as possible. The mgh/L dosage, Ct product achieved, was calculated from the FID results for each exposure.

Treated material and untreated controls spores of *T. indica*, *T. controversa* and *U. maydis* were seeded onto water agar medium to assess viability based on spore germination. Treated oospores were mixed into the upper 5 cm layer of soil in a 2 X 2 inch plastic pot and planted with seeds of a highly susceptible sorghum cultivar and placed in a growth chamber for disease development.

Results and discussion

Figures 1 to 3 present the response of treated spores to the range of doses and temperatures. These data indicate that naked teliospores of the three smut fungi (*T. indica, T. controversa* and *U. maydis)* were more easily controlled than spores still contained within the fungal structure or sorus of *T. indica* and *T. controversa*. Spores that were dusted onto corn were the most difficult to control. This would indicate that surface interactions on the corn seeds and penetration of EDN into the fungal structures reduce the effective dose.

All three smut species treated at 22°C were controlled to a high level at dosages less than 2000 mgh/L. As this is the likely treatment temperature of the commodity and the proposed dosage would be greater than this experimental treatment, using EDN should provide good control of these pathogens.

In general the data indicate that EDN was more toxic at higher temperatures. Overall, *T. indica*, with its large teliospore, was the most tolerant of the smut fungi.

Oospores of *S. sorghi* germinate poorly, if at all, on artificial medium hence this was not a feasible method to check efficacy for this pathogen. No vital stains were shown to be effective with oospores of *P. sorghi*. However, the treated oospores mixed into soil and planted with seeds of susceptible sorghum also proved problematic as an assessment of efficacy. Trace infection was observed in the untreated controls, and in one replicate of the 1 hr treatment at 17°C at a dose of 120mgh/L. No other infection was recorded in the remaining 44 treatments. However, cross-contamination of the treated oospores cannot be ruled out. Most likely, given that initial inspection of the infected material indicated a high number of spores, is that the newly acquired oospores may have been exhibiting yearly season dormancy, which would explain the low levels on infection in the inoculated control plants and near absence of infection in any of the treatments (Pratt, 1978[7]).

References

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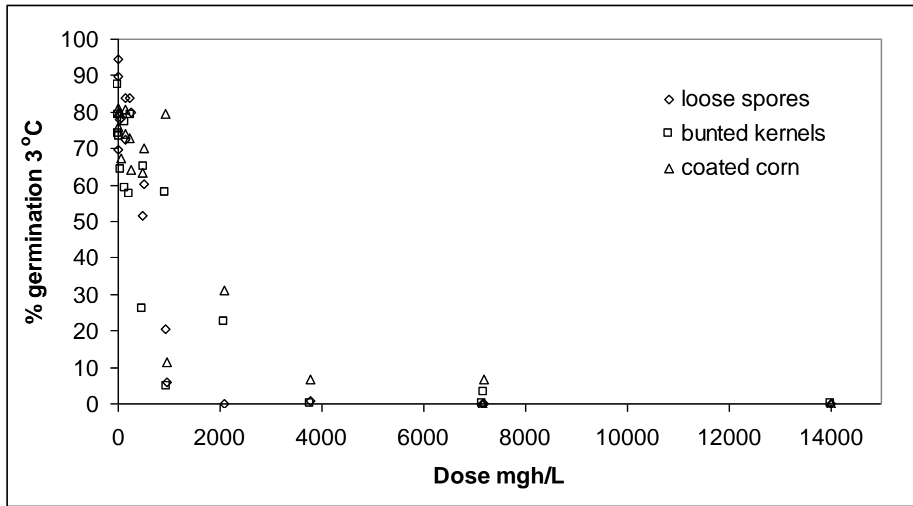
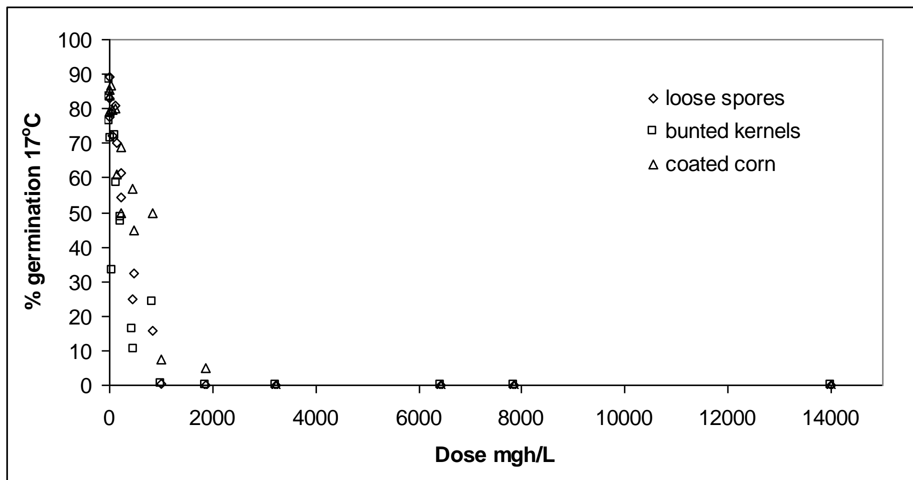




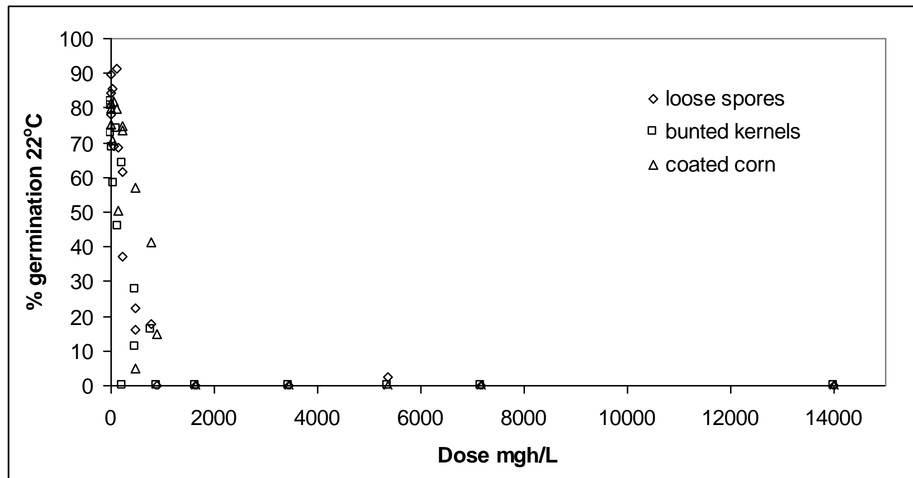


Figure 1. Efficacy of ethanedinitrile (C_2N_2) at 120 mg/L against teleospores of *Tilletia controversa* treated as loose spores, bunted kernels and spores dusted onto corn for 10, 25, 60, 120, 250, 500, 1000, 1750, 3000, 4500, 7000 minutes of exposure at 3, 17 and 22°C

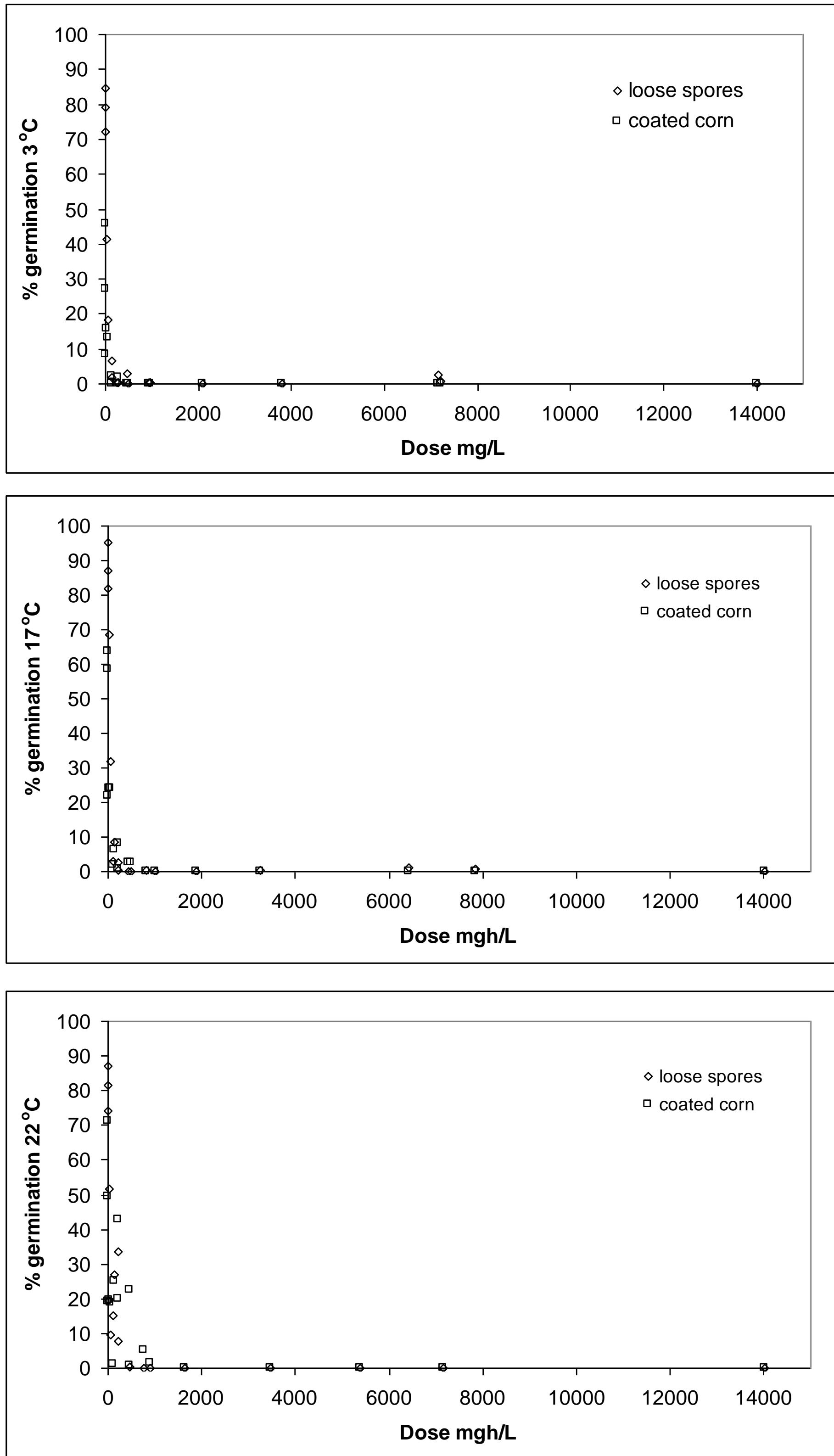
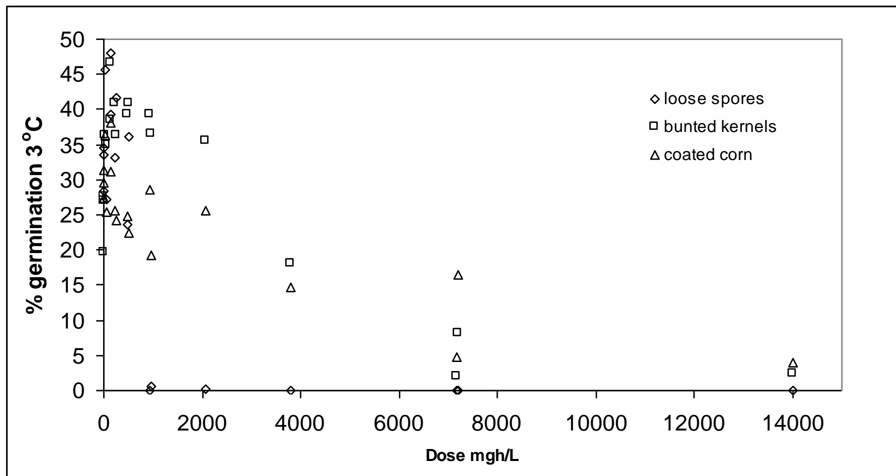
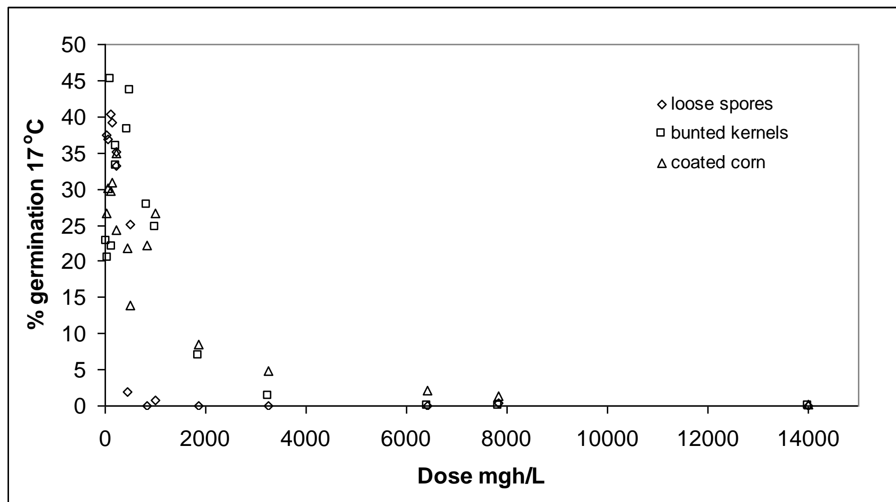


Figure 2. Efficacy of ethanedinitrile (C_2N_2) at 120 mg/L against teleospores of *Ustilago maydis* treated as loose spores and spores dusted onto corn for 10, 25, 60, 120, 250, 500, 1000, 1750, 3000, 4500, 7000 minutes of exposure at 3, 17 and 22°C





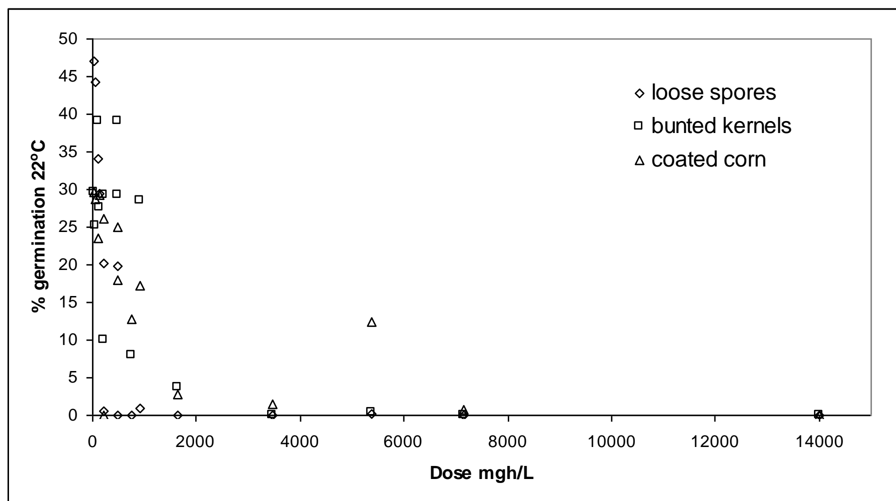


Figure 3. Efficacy of ethanedinitrile (C_2N_2) at 120 mg/L against teleospores of *Tilletia indica* treated as loose spores, bunted kernals and spores dusted onto corn for 10, 25, 60, 120, 250, 500, 1000, 1750, 3000, 4500, 7000 minutes of exposure at 3, 17 and 22°C

6. Small scale trials (50kg)

Aims of the 50 kg trials

The primary objective was to demonstrate the ability to fumigate target commodities. This is important, as the commodities are an important link for exotic pathogens. Viruses in particular are not considered to be a viable target for fumigation; however by killing the host the life cycle of a virus may be interrupted. Similarly for obligate fungal pathogens the absence of the host is an additional level of control over any direct fumigant action on the pathogen. The trails consist of fumigation of 50 kg lots of maize, wheat and barley. This will provide initial commodity interactions with EDN to assist in design of a commercial application protocol to ensure target doses are achieved throughout a treatment.

Methods

Commodities were equilibrated to 14% moisture content prior to fumigation and available water (A_w) estimates measured using an AquaLab CX-2 water activity meter (Decagon Devices, Inc., Washington, USA).

Prior to treatment, EDN was transferred from a gas bottle in a Tedlar bag and analysed to determine the percent purity using a Gas Density Balance, Tracor 220 M (Tracor Inc., Austin, TX, USA) with a 1 m × 5 mm glass column packed with Porapak Q 100/120 mesh (Alltech Associates, Deerfield, IL, USA) run at 105°C and a carrier gas (N_2) flow of 150 mL min^{-1} using 1,1,1,2 tetrafluoroethane as a reference gas. The quantity of gas needed to achieve the target concentration was calculated after correcting for pressure and temperature. Based on these calculations air was withdrawn from the drum and replaced with an equivalent volume of EDN.

The drum was incubated at controlled temperature for a period of 5 d. Headspace concentrations were measured immediately prior to each top up dose. As the headspace concentration fell rapidly immediately after application, sampling was most intensive around this time. Analysis by gas chromatography (GC) was by a flame ionisation detector (FID) equipped Shimadzu GC6AM (Shimadzu Seisakusho, Kyoto, Japan) on a 1 m × 3 mm glass column packed with HaysSep Q (Alltech Associates, Deerfield, IL, USA) run at 80°C and with a carrier gas (N_2) flow of 40 mL min^{-1}.

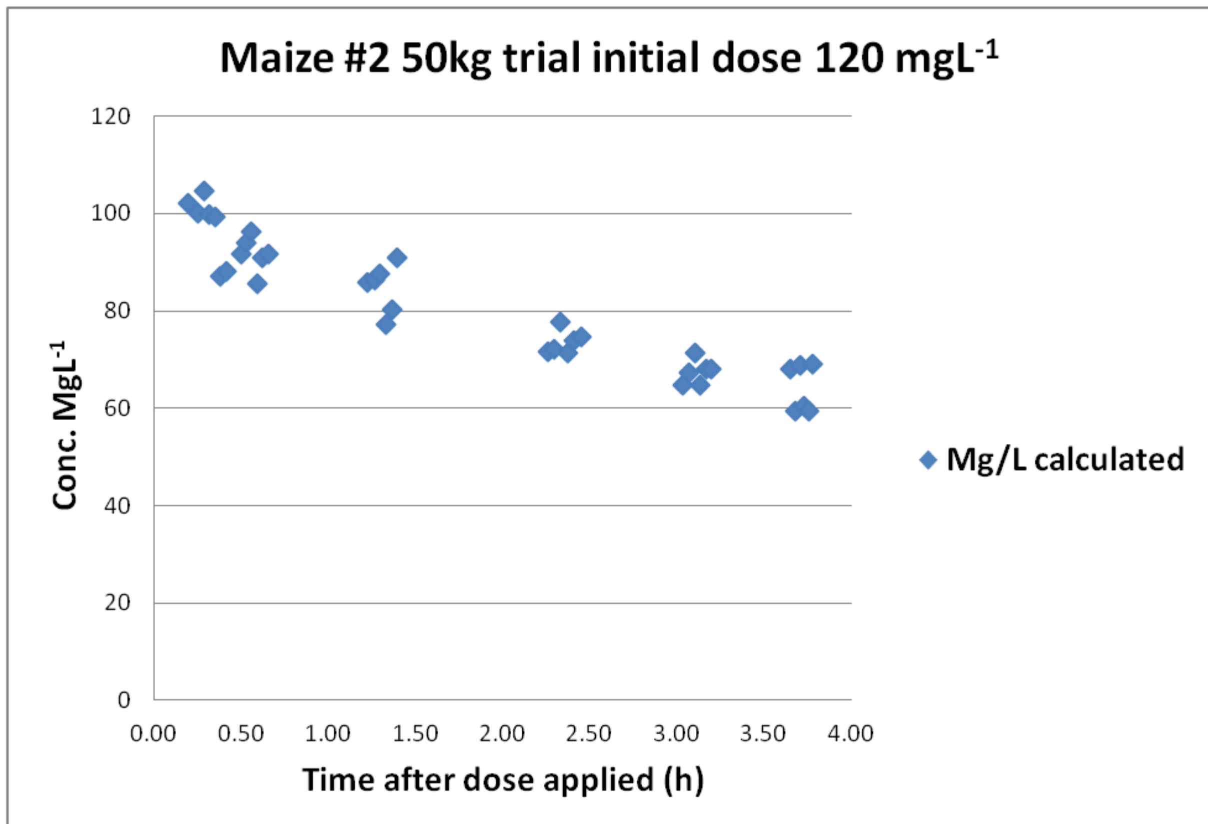
Cumulative concentration by time was calculated and the exposure terminated when the target ct was reached. At the completion of the experiment the contents of the container were aired prior to viability assessment.

Germination assessment was conducted according to International Seed Testing Association methods. Accordingly each of 400 seeds for each commodity dose combination was assessed as either normally germinated, low vigour, abnormal, fresh (normal in appearance but non-germinated), mouldy, hard (not imbibed), or empty. Germination test procedures are described in Appendix C of FLOT.124.

Results

The second 50kg maize trial showed that the loss of EDN was rapid as shown in Figure 1 with more than 50% gas sorbed or broken down in less than 5 hours. With the target concentration of 120 mgL^{-1}, this would not provide enough dosage for a daily top-up for a five day exposure period.

Figure 1 shows the rapid loss of concentration



This result was similar for all commodities tested when the attempts to replace gas lost failed.

Discussion

The experiment was structured to identify differential sorption over time. The multiple doses applied in five applications over five days with measurement of C2N2 headspace concentrations over time did not achieve high dosages because of the rapid loss of EDN with time. Attempts were made to continuously replace gas sorbed and lost to breakdown by attaching a Tedlar bag to the recirculation of gas through the fumigation chamber. This was not as successful as hoped due to the stringent OH&S requirements of working with EDN. It may also have resulted from a less than gastight system as we were relying on the negative pressure of the depleted system to draw in the lost gas from the Tedlar bag. Daily incremental injections to replace gas lost remained the main method of dosing. As a result dosages were not well controlled in this phase of the work. However, all commodities were devitalised and the importance of maintaining the concentration at or near $120 mgl^{-1}$ demonstrated. This carried over into the design of the 1 tonne trials.

References

FLOT.124: Devitalisation of imported feed grain by fumigation Brendan J Smith, Rainer Reuss, Colin J Waterford, David P Rees, Sarwar Muhammad, Daphne A Mahon and Yonglin Ren.

7. Medium scale trials (1 tonne)

Medium scale trials treating one 500kg and four 1 tonne batches of maize with EDN where completed.

Methods

A one tonne sealed bin fitted with a recirculation system is shown in figure 1. This allowed for rapid application of a weighed amount of EDN to be piped to the base of the cone into the recirculated flow from a cylinder placed on a set of digital scales. The target concentration was chosen to keep the concentration below the flammability level of EDN. Once the target concentration of 120 mg/L was applied a maintenance flow was set via a flow meter to replace gas lost through sorption and breakdown to maximise the applied dose over the five days of treatment.

The bin was fitted with a number of sampling points to assess distribution within the bin and that all grain treated received an equivalent dose.

The percent purity of the cylinder of EDN was analysed using a Thermal Conductivity Detector (TCD) fitted to an SRI model 8610C gas chromatograph using a 3 foot 1/8 inch column packed with Porapak Q 80/100 mesh, run at 100ºC with a carrier gas (He) 20 mL-1. This was used to prepare three 500mL gas standards in 1L Tedlar gas sampling bags to calibrate the response of a nitrogen phosphorus detector (NPD).

Gas distribution and measurement of dosage applied was assessed by measuring the concentration at a number of points throughout the bin by injecting samples with gastight syringes onto the FPD.

The bin was plumbed with sampling points on the eastern and western side top middle and bottom edge; in the centre of the bin; in the free air space above the maize at the top and in the bottom cone.

Figure 1. Sealed bin and recirculation system for treatment of one tonne of maize with EDN

Figure 2, GC data system and Tedlar sampling bags with gastight syringe for injecting samples onto the GC.

Results and discussion

Figure 1 shows the layout of the one tonne sealed bin and the recirculation pump. The cylinder of EDN is in the cage situated on a digital scale for weighing in the initial dose and monitoring the amount of EDN continuously added via the rotameter on the left-hand side of the cage.

Figure 2 shows the GC and Tedlar sampling bags.

Figure 3 show the dosage of the feeder trial across the five days exposure. Apart from the initial over dose at the beginning, the system of replacing the gas lost appears to work. The steady rise towards the end may indicate the sites causing breakdown are being used up. This phenomenon was noticed in FLOT.124 for maize and was one of the reasons for selecting this commodity as the target commodity, as less gas would be required to achieve a target dose.

Table 1 shows the level of devitalisation of five trials of maize treated with EDN

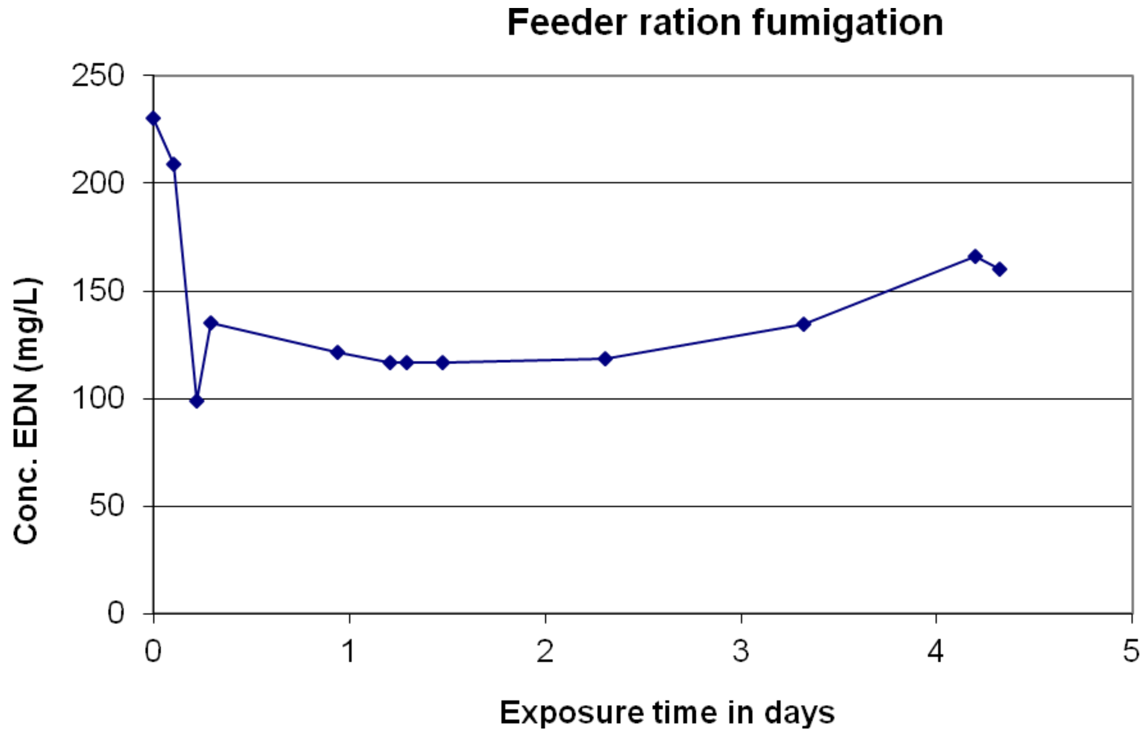


Figure 3 Exposure of 500kg maize to EDN with continuous top up of gas lost to breakdownand sorption.

Table 1 devitalisation of maize treated with EDN in a sealed silo over five days

Trial	Replicates 4x400 seeds	% Germinated	% Ungerminated
500 kg feeder trial	**control**	89.5	10.5
	Top	**0.0%**	**100.0%**
	mid	**0.0%**	**100.0%**
	bottom	**0.0%**	**100.0%**
1st 1 tonne maize	**control**	**94.1%**	**5.9%**
	Top	**0.0%**	**100.0%**
	mid	**0.0%**	**100.0%**
	bottom	**0.0%**	**100.0%**
2nd 1 tonne maize	**control**	**96.0%**	**4.0%**
	Top	**3.0%**	**97.0%**
	mid	**1.3%**	**98.8%**
	bottom	**0.0%**	**100.0%**
3rd 1 tonne maize	**control**	**97.1**	**2.9**
	Top	**0**	**100**
	mid	**0**	**100**
	bottom	**0**	**100**
4th 1 tonne maize	**control**	**96.5**	**3.5**
	Top	**0**	**100**
	mid	**0**	**100**
	bottom	**0**	**100**

8. Commercial scale trial (500 tonne)

Commercial scale trials (500 t) with maize (barley or wheat to be substituted for maize at discretion of MLA) are designed to complete the final large-scale EDN test, with extensive germination testing of treated commodity and gas analysis to assess sorption. These trials will provide the final data set on a protocol for devitalisation of imported maize for sign off by Biosecurity Australia.

Protocol for commercial-scale trial devitalisation of maize with ethylene dinitrile

The trial protocol is based on experience gained from one tonne trials of EDN on maize and is designed to investigate the following major areas:

- Application methods to maintain a high but safe level of EDN in the fumigation structure for the shortest feasible time
- Demonstrate devitalisation of treated maize by sampling selected locations throughout the treated bulk
- Assessment of sorption and breakdown of EDN on maize (dose)
- Time taken to breakdown EDN after stopping application of EDN
- Necessity for venting residual EDN from treatment atmosphere and maize at completion of treatment
- Assessment of EDN residues remaining in treated maize
- Measurement of levels of EDN in the risk area and bystander environment during application / aeration / subsequent out-loading

The trial is designed to ensure compliance with the following specific conditions:

- At out-loading, the in-bin concentration of EDN must remain below the Threshold Limit Value (TLV) of 10 ppm

- The fumigation is to be supervised by licensed fumigators. The trial to be conducted by competent scientist and technicians skilled in gas analysis germination and trial design.

Materials and application

Two gastight silos located at the GrainCorp site at Fisherman Islands, Qld or similar would be suitable for the trials. They are of steel construction, with 60 m^3 (50 tonne capacity) and are self out-loading with a cone at the top and bottom (Figure 1). The silos are equipped with a recirculation system for phosphine or methyl bromide fumigation consisting of a PVC pipe (10cm internal diameter, i.d.) running from the top of the silo to the ground where they are connected to a common recirculation fan (0.5 kW).

The maize used in this trial to be supplied by MLA. The moisture content will be adjusted to 14% by addition of moisture at in-loading and aeration to distribute and equalise moisture content.

The EDN to be used is the formulation Sterigas®, supplied by BOC Australia and has an active ingredient of >99.5% EDN. Application would be initially by weight via a gastight connection into the recirculated airflow of the sealed silo to establish a concentration of 120 mg L^{-1} then a maintenance flow to replace EDN sorbed and broken down by the maize. The

amount of EDN required for each trial is expected to be 2.5kg tonne^{-1} or about 125kg for the target cxt of 6000mg.hL^{-1}.

A significant part of the initial trial is to assess the best commercial method to maintain a relatively steady concentration of 120 mg L^{-1} throughout the treatment period. This concentration is selected as being below the flammability lower explosive limit (LEL) for EDN but sufficiently high to achieve the dose required in the shortest exposure time. One method would be to measure the concentration periodically and add pulses of EDN when the concentration falls to say 115 mg L^{-1} to raise the concentration to say 125 mg L^{-1}. This could be automated with suitable analytical equipment. Another method using the same analytical equipment would be to establish a feedback loop to control the flow of EDN into the recirculation to maintain the concentration at 120 mg L^{-1}.

The application of EDN to the silo will be through a gastight gas line connected to the recirculation duct. With a high level of seal on the bin gas loss from the bin during fumigation should be minimal.

Devitalisation of maize

Prior to the application of EDN, untreated control maize samples will be taken from the grain bulk surface (approximately 2 kg) and, using a sampling probe, from the centre of the silo to the depths of 1, 2 and 4 m (approximately 200 g from each depth).

At the end of the fumigation the top hatch is to be opened and post treatment grain samples taken using the same procedure. Ten out-loading samples of 200 g will be taken from the bottom of the silo at timed intervals (5-6 min), freighted to the laboratory for germination tests.

Grain temperature and in-bin air conditions

During the fumigation, grain temperature, headspace air temperature and relative humidity will be automatically recorded in the silo using a HOBO® data logger unit, (Model number H08-004-02, Onset Computer Corporation, MA 02532, USA, www.onsetcomp.com). To measure grain temperature, the HOBO probe sensor was inserted 1 m below the grain peak in the centre of the silo (Figure 1). To measure the headspace air temperature and relative humidity, the HOBO® was hung 0.5 m above the grain peak in the headspace of the silo (Figure 1). The recorded data were read with the software BoxCar® Version 3.6+ for Windows (Onset Computer Corporation,). The HOBO® sensors will be previously calibrated in the laboratory against an alcohol filled glass thermometer, a range of glycerol/water solutions for relative humidity and against each other.

Sampling maize during treatment

If possible several maize sampling ports should be installed at the base of the bin to take progressive dosage samples to track the progress of devitalisation. Samples taken would be aired and then stored for germination.

Alternatively samples can be taken at 4000, 6000, and 9000 mg h L^{-1} by interrupting the dose and taking samples from the surface and running a small sample from the base. Samples aired and stored for germination.

Measuring in-bin concentrations of EDN

The silo will be fitted with 9 sampling lines (nylon, 3 mm internal diameter) which lead to a sampling position outside the fumigation area (8 m from the silos). The locations of gas

sampling ports are shown in Figure 1. Samples will be drawn with an electric pump and either taken directly to analytical device or stored in Tedlar® gas sampling bags (1 L) until analysis, usually within 1 hour of sampling (Figure 2).

The concentration of EDN to be determined using a SRI 8610C portable gas chromatograph (GC) equipped with a nitrogen phosphorus detector (NPD) after isothermal separation on a HaysSep column with the oven temperature set at 110°C. Concentrations of EDN are calculated on the basis of peak areas against external standards, prepared by dilution in sealed 250 mL bottles using the pure EDN as the standard using the same HaysSep column. A sample volume of 50-100 µL was injected into the GC-NPD. Purity of the EDN source standard is checked using a thermal conductivity detector (TCD)

Measuring environmental levels of the fumigant during fumigation

For these trials general environmental and occupational health and safety (OHS&E) will be according to the protocols established by BOC Australia

Environmental air samples to be taken downwind during application and exposure period with a 1 L syringe, in triplicate at head height, at distances of 3, 6 and 15 m (Check distances) from the base of the silo. Gas samples to be stored in Tedlar® bags before analysis, usually within 1 hour of sampling. Chromatographic conditions for analysis of EDN concentrations are as previously described, although on a more sensitive setting and a larger volume of gas (100-200 µL) to be injected into the GC. Some permanently placed gas sampling lines will be measured with a Spectroscopic analyser supplied by BOC Australia.

Analysis of EDN residues

Residues of EDN in treated maize to be analysed following the procedure as described by Ren.

Moisture Content

Moisture content of maize samples to be measured using the oven method as described in the International Organisation for Standardisation, method 712 (ISO 1985).

Discussion

Arrangements for access to sufficient quantities of EDN for the trial are not complete at this stage

9. Recommendation

The use of EDN alone to fumigate imported maize will cover many but not all quarantine concerns. The remaining outstanding issue is control of associated weed seeds. Not all weed seeds are devitalised and a number remain untested because of availability or failure of untreated controls to germinate. Hence a protocol similar to that set out in the importation of Sweetcorn seed from Idaho USA for sourcing and cleaning the shipment of maize prior to importation may be acceptable to Biosecurity Australia as the first step in a fumigation based protocol. In addition the shipment would be essentially free of insects and pathogens. It is then recommended that a single treatment with EDN at a quarantine facility at point of importation can be designed to cover the remaining quarantine issues by:

- devitalising the commodity
- killing any pathogens that present as a contaminant of grain handling pathways through which the shipment would need to pass as it is loaded onto ship
- controlling all insects, associated with international trade of grain in the delivery pipeline, that may be a picked up.

As weed seeds will be excluded from the shipment by physical means it is recommended that the dose is set at approximately 6000 mg h/L EDN to control the remaining risks. This would be sufficient to devitalise the maize control any contaminant pathogens picked up during handling and transport and control all insects that may be present. As this is less than half the dose applied in the one tonne trials, it would significantly reduce the cost of EDN applied and the fumigation time, improve the logistic capacity of treating large bulk consignments, and provide additional options for application (e.g. periodic or pulsed addition) rather than continuous application of EDN.