The role of germination in the assessment of the potential weediness of Bt cotton (*Gossypium hirsutum* L.) in tropical Australia

R.J. Eastick[1] and M.J. Hearnden[2]

[1]Department of Business, Industry and Resource Development and Australian Cotton Cooperative Research Centre, Northern Territory AUSTRALIA

[2]Department of Business, Industry and Resource Development, Northern Territory AUSTRALIA

Correspondence author rowena.eastick@nt.gov.au

ABSTRACT

There is a demand from scientific, regulatory and environmental bodies to evaluate the risks associated with the release of genetically modified crop plants. One identified risk was that cotton genetically modified for insect resistance may have additional fitness that could confer increased weediness for Bt cotton in tropical Australia. There are two approaches to directly assess the ecological risk of transgenic crops; large-scale demographic experiments, and targeted experiments to detect the effect of the transgene during specific life stages. Two targeted experiments were conducted to evaluate parameters critical to the success of cotton that may have unintentionally dispersed from a production area and established as a volunteer plant, or 'weed', and whether this was different between Bt cotton and its non-transformed counterpart. The experiments were conducted at Kununurra, Western Australia, and complemented a large-scale ecological study, which examined potential weediness of Bt cotton over a range of habitats in northwest Australia. The experiments were designed to investigate; 1) Germination rates of 3 seedtypes (black seed, fuzzy seed and seedcotton) for each of 3 genotypes (Cry1Ac, Cry1Ac+Cry2Aa and non-transformed) of cotton seed when buried or left remaining on the soil surface, and; 2) Survival rates of 3 seedtypes for each of 3 genotypes of cotton seed when left remaining on the soil surface over a dry season (winter). There was a highly significant seedtype by soil coverage interaction (P<0.001) on germination, with the buried treatment of all seedtypes greater than seed remaining on the soil surface. Buried seed cotton had significantly lower germination compared with the buried black seed and fuzzy seed. This is important for weediness, as seed cotton is the type most likely to be dispersed, but coverage of seed by soil would be reliant on a chance event such as rainfall. There were no viable seed remaining after exposure in a native bush habitat over a single dry season, attributed to predation by invertebrates and small mammals. Germination and seed survival are important demographic stages in the establishment of volunteer cotton plants. There was no effect of genotype on either of these two parameters within these targeted experiments, indicating that the addition of the Bt gene does not pose additional fitness for germination and seed survival.

Introduction

There are a number of concerns regarding the impact on the environment of the adoption of genetically modified (GM) crops. Recent debate on transgenic DNA introgression into traditional Mexican maize landraces has highlighted the controversy which often surrounds the release of GM crops (Quist, 2001; Christou, 2002) and has reiterated the demand that currently exists for ecological biosafety research prior to the release of these crops (Saeglitz and Bratsch, 2002).

Bt (*Bacillus thuringiensis*) cotton containing the Cry1Ac gene (INGARD®) was the first transgenic crop to be commercially released in Australia in 1996, with regulatory guidelines restricting its production to the temperate regions south of the 22nd parallel. The potential availability of land and water combined with new knowledge and production technology has stimulated increased interest in growing cotton in northern Australia (Yeates, 2001). In 2002, the submission for commercial release throughout Australia of BOLLGARD II®, containing the Cry1Ac and Cry2Ab genes was successful, although specific conditions on the release of Bt cotton north of latitude 20°South (northern Australia) were imposed due to concerns over possible weediness (www.ogtr.gov.au/rtf/ir/dir0012finalrarmp.rtf).

The Office of the Gene Technology Regulator (OGTR) is Australia's regulatory body dealing with the release of genetically modified organisms. They suggested investigating if the addition of the Bt gene would give an ecological advantage that would increase weediness over non-transformed cotton in tropical regions. Northern Australia is a region characterised by a wet-dry tropical climate (Williams *et al.,* 1996) and *Gossypium hirsutum* evolved in tropical and sub-tropical areas (Fryxell, 1979). Naturalised populations exist in northern tropical Australia. Increased weediness could occur through the Bt gene enhancing the ability for improved cultivars to become naturalised, or by transgression into existing naturalised populations and altering their ability to persist. It was a requirement from OGTR, in conjunction with Environment Australia (EA) and community demand, to evaluate the potential weediness of Bt cotton prior to any commercial release in northern Australia.

Linder and Schmitt (1995) outline two approaches to directly assess the ecological risk of transgenic crops; large-scale demographic experiments which examine the entire life history over several years; and smaller, targeted experiments which examine life history phases when the transgene is most likely to have an impact. A large-scale multi-site study was established in the 1999-2000 wet season (summer) to examine a number of demographic traits over several years (germination, survivorship and fecundity), as contributors to invasiveness of Bt cotton over a range of habitats in northern

Australia. Results from this study are reported elsewhere (manuscript in preparation), but germination was identified as a stage critical to the establishment of volunteer cotton populations. Factors influencing germination were particularly important in non-agricultural production habitats, where seed would not be introduced into prepared ground conducive to maximum germination.

There are three forms of seed by which cotton can disperse into the environment and germinate, namely black seed, fuzzy seed, or seed cotton, each with differing probabilities of introduction into different habitats at varying times throughout the year. For this study, germination of the three seedtypes was assessed to allow for potential dispersal under different scenarios.

The greatest chance of non-intentional cotton seed dispersal is as seed cotton during the harvest period, either as residue in the field after picking, drift into drains and paddock verges, or spillage onto roadsides during transport to the gin. This coincides with the onset of the wet season (October-November) and available moisture for germination. Seed cotton may also be dispersed over the dry season (winter), as perennial volunteer cotton plants mature fruit over this time. The greatest chance of intentional cotton seed dispersal is as fuzzy seed introduced as cattle feed, usually fed as a supplementary feed over the dry season. Seed spilled from feed troughs can lead to establishment of volunteer plants. There is also evidence that a proportion of seed can remain viable after passage through the digestive tract (Bolam, personal communication). The chance of unintentional dispersal of black seed is extremely low, but could occur through seed spillage when sowing at the commencement of the cotton production season in the early dry season.

Two targeted experiments examined the effect of seedtype and genotype on germination; firstly, when seed was exposed to different soil coverage conditions, and secondly, when seed remained on the soil surface for different periods of time.

Seed dispersed over the dry season (May-September) will remain exposed on the soil surface until the next rainfall or irrigation event, which could stimulate germination. The first experiment aimed to simulate seed dispersal during a dry season and evaluated changes in seed viability until the onset of the following wet season. Differences in seed viability would indicate the probability of germination and establishment of volunteer cotton plants and the potential weediness between genotypes.

Cotton seed germination in the field has primarily been assessed under conditions where seed is covered by soil. In 'escape' conditions, it is more likely that the seed would remain on the soil surface, for example on road verges. The second experiment aimed to compare germination among the three seedtypes and three genotypes to quantify differences when seed was buried, or on the soil surface, and how this affected germination, and thus weediness.

This paper presents findings from these two targeted experiments which evaluated germination differences between Bt cotton and conventional cotton as a specific demographic phase fundamental to potential weediness. These results complemented a large-scale multi-site study that compared demographic traits as components of weediness between Bt and conventional cotton over a range of habitats.

Experimental Procedure

Experiment A: Seed survival over a dry season

The site was a native bush habitat on Cununurra clay (Williams *et al.*, 1985) at Kununurra, Western Australia (15°39'S, 128°42'E). The experiment was designed as a three way factorial randomised complete block, with factors: genotype (Cry1Ac, Cry1Ac+Cry2Aa and non-transformed), seed type (black seed, fuzzy seed and seed cotton) and time of seed exposure (seven days, 28 days, three months and five months). Seeds were sown during the dry season, on 28 July 2000. Twenty-five seeds per plot were encased in 1 mm flymesh netting measuring 10 cm by 10 cm, which was placed on the surface of a small hand-cleared area, then secured with wire pegs. Plots were 2 m apart. The nets containing the seeds were collected after each of the four time periods, corresponding to early August, late August, late October and mid-December respectively. Seed viability was then evaluated through a laboratory germination test. Seeds representative of each of the field treatments were also stored in the laboratory and subjected to an un-replicated germination test coinciding to the times of each collection of the seeds from the field for comparison.

Experiment B: Germination between buried and non-buried seed

The experimental design used was a split-split-plot with seed type (black seed, fuzzy seed and seed cotton) as the main plot treatment, genotype (Cry1Ac, Cry1Ac+Cry2Aa and non-transformed) as the sub-plot treatment, and depth (buried and surface) as the sub-sub-plot treatment. The site was a cleared area within a native bush habitat on cockatoo sand (Williams *et al.*, 1985) at Kununurra, Western Australia. One hundred seeds were hand-sown in 25 cm by 25 cm plots 2 m apart. Soil was removed to a depth of 4 cm, seed pressed gently into the disturbed soil, then covered with the original soil for the buried treatment. Seed was placed on the cleared soil surface for the surface treatment. Seed was sown on 10 February 2000, hand-watered after sowing to simulate rainfall, and emerged seedlings watered to the time of predicted first square,

equivalent to approximately 540 DD12 (Constable and Shaw, 1988). Emergence was recorded at 4 days after sowing (DAS), 11 DAS and 18 DAS.

Differences in germination success for both experiments were analysed with a generalised linear model (GLM) using binomial errors and a logit link (McCullagh and Nelder, 1989). Pairwise comparison tests to discriminate between treatment means were done using Tukey's Tests and adjusting the type 1 error rate with the Dunn-Sidak method (Ury, 1996). Analyses for Experiment A were conducted only for the 7, 28 and 90 days exposure measurements.

Results

Experiment A

There was a significant interaction between time of exposure and seedtype (P=0.006, Table 1). Fuzzy seed had a higher germination rate than black seed and seed cotton up to 28 days, but after 90 days of exposure in the field, seed cotton germination rates were relatively higher, although germination of all three seedtypes was extremely low. Importantly, there were no viable seeds remaining for the field treatments by the time final measurements were conducted at 150 days. Point estimates for the laboratory-stored illustrate the overall relative reduction in germination values for the field seed treatments over time (Figure 1). There was also a significant interaction between genotype and seedtype (P=0.015, Figure 2), with the germination of seed cotton lower than the other two seedtypes for the conventional genotype only. Point estimates for the laboratory-stored seed illustrate the overall reduction in germination values for the field seed treatments.

Experiment B

There was a significant seedtype by depth interaction (P<0.001, Table 2). The germination rate of buried seed cotton was significantly lower than either buried black or fuzzy seed types. The germination rate of seeds on the surface was low for all seedtypes (mean 17%), though post-hoc comparison tests indicated that black seed was significantly lowest.

Germination for all seedtypes was significantly lower for the surface treatments than the buried treatments (Figure 3). Although the three-way interaction result was significant (P=0.045), there appears to be no pattern attributable to genotype for any of the seedtypes at either depth (Figure 4).

Discussion

In commercial production, the establishment of an adequate number of vigorous seedlings is important because it is the first phase of the production cycle that sets a limit on yield potential (Wanjura, 1981). This also has relevance for evaluation of weediness, where

germination is the crucial initial phase leading to establishment of volunteer cotton populations, particularly in non-production habitats. There was no difference in germination between transgenic and conventional genotypes of the same parent line under commercial production conditions (Serdy and Berberich, 1995) and our conclusions support these results under sub-optimal conditions for germination.

Serdy and Berberich (1995) discuss that cotton is not considered to possess seed that can persist in the environment for long periods of time. Results from Experiment A confirm this. There were no viable seeds remaining by the time of final measurement at 150 days, demonstrating that the Bt gene does not provide additional fitness that would produce more persistent seed in natural habitats. At the 90 day measurement, the mesh containing the seed was observed to have been chewed, and there was evidence of chewing on most seed. The marked decline in seed viability from 28 to 90 days for the field seed treatments but not the laboratory stored seed was thus attributed to insect predation, or from small animal foraging. Seed dispersed through the dry season and exposed on the soil surface is unlikely to germinate and pose a weed threat at the commencement of the wet season, which may be up to 180 days after the seed has dispersed.

The interaction between seedtype and genotype, where there was a decline in field germination of seed cotton for the conventional genotype, could be due to differences in parent plant history in the field, and the culling of low density seed in the acid delinting process. All three seedtypes were sourced from a field experiment that was managed for insects as an Ingard® crop. Conventional cotton may have suffered greater insect pressure, consequently producing damaged seed within each boll (personal observation). Open bolls from each genotype were hand-harvested in October 1999, and seed cotton was then small ginned (for fuzzy seed) and acid delinted (for black seed) in small batches. Damaged black seeds of all genotypes would be culled during the delinting process, but this process would not exclude damaged fuzzy seed and seed cotton, higher for the conventional than the transgenic genotypes. Insect management in the field from which seed may eventually disperse has ramifications on the probability of germination. Conventional crops managed as low input fields as a component of insect resistance management strategies may have lower overall risk of dispersal of viable seed than transgenic crops.

For Experiment B, the result that germination for all seedtypes was significantly lower for the surface treatments than the buried treatments was as expected, and supported the importance of seed soil contact for germination. This was discussed by Kerby *et al.* (1996) who concluded that intimate seed-to-soil contact was critical, especially in sandy soils, regardless of initial moisture content, and Wanjura *et al.* (1969), who omitted shallow planting depth treatments within a plant-

ing depth experiment, because of erratic emergence due to soil drying. Seeds that germinate at the surface would be less likely to survive than those deeper in the soil because of the greater risk of death by desiccation or disturbance (Colosi *et al.,* 1988). Ants (native argentine black ants and meat ants, *Iridomyrmex* spp.) were observed to eat seed exposed on the soil surface. The lower germination of black seed than seed cotton and fuzzy seed on the surface is indicative of the insulation and protection afforded by the lint, from both predation and desiccation.

There is scant available literature on seed cotton germination, likely due to the crop being grown solely for the purpose of removing the lint, and not being an appropriate form for planting seed. Plant stand success is influenced by soil impedance (the resistance to root or shank elongation), which determines how hard the seedling shank must push on the cotyledons to move them through the layer of soil. Although some soil impedance is beneficial for shedding the seed coat from the cotyledons, severe impedance can restrict the plant's ability to emerge (Kerby *et al.,* 1996). The lower germination of seed cotton than black and fuzzy seed when buried was attributed to obstruction of the emergence of the cotyledons by the cotton fibers, as had been observed on the soil surface, and greater difficulties to push the lint through the soil.

The low germination of all seedtypes on the soil surface has implications for weediness. The initial establishment of transgenic organisms depends to a considerable extent on pure chance (Tomiuk and Loeshcke, 1993). Under conditions of unintentional dispersal, seed would fall onto the soil surface, or standing vegetation, and would require a chance event such as soil wash by rainfall to be covered to increase the probability of germination. Thus the chances for establishment of volunteers from seed on the surface would be low.

Seed and dispersal ecology are major determinants of weed fitness and population growth rate (Jordan, 1999), where seed bank dynamics and seedling establishment may be particularly important for the potential persistence of escaped transgenes (Schmitt and Linder, 1994). The ability of seeds to remain viable and dormant in the soil and to germinate in the presence of environmental cues that indicate a locally favourable environment for growth and reproduction can be stronger determinants of fitness than selective pressures during vegetative and reproductive phases (Linder and Schmitt, 1995). Although the benefits of the Bt gene are largely allocated to the reproductive structures of the cotton plant, germination is a vital component in the establishment of volunteer, or 'weedy' cotton populations. Our results show there was no effect of genotype on germination, except as an interaction likely related to parent seed source history, demonstrating that the addition of the Bt gene does not infer additional fitness by providing protection to cotton seeds that would increase germination compared to conventional genotypes.

Conclusion

There is no evidence to suggest that the addition of the Bt gene would not increase weediness by affecting germination. Fuzzy seed has the best chance of germination when exposed on the soil surface for a limited period of time due to some protection from predation and desiccation afforded from the fuzz, but little inhibition of emergence of the cotyledons. However, for all seedtypes, it is unlikely that seed exposed on the soil surface over a dry season would germinate and pose a weed threat at the commencement of the following wet season.

It is important to note that germination is only a component in the entire life cycle of a perennial plant, albeit it an important one. Conner (2003) states that *"Changes in the rate of a single demographic process cannot be taken alone as a measure of plant invasion, a methodological concern often overlooked when enhanced invasiveness of GM crops is considered"*. This was recognised in the implementation of a complementary large-scale multi-site experiment, where invasiveness was defined as a component of weediness, and was evaluated as the population change over time, l, consistent with discussions by Crawley *et al.* (1993) and Kareiva *et al.* (1996). In agreement with Conner (2003), the evaluation of l is more valuable than estimates of a single demographic process (i.e. performance at any particular life history phase) which provide no information about invasiveness when used in isolation. However, determining that the Bt gene does not provide additional fitness over conventional genotypes for germination is an important precursor to the overall assessment of potential weediness.

Acknowledgments

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Table 1. *Analysis of deviance (ANODEV) table on logit-transformed proportion of germinated seeds for genotype, seedtype and time of exposure.*

Source	df	Deviance	F	P
Genotype	2	8.861	2.729	0.074
Seedtype	2	11.250	3.465	0.038
Time	2	1179.313	363.178	<0.001
Genotype x Seedtype	4	22.193	3.417	0.015
Genotype x Time	4	3.287	0.506	0.731
Seedtype x Time	4	26.338	4.056	0.006
Genotype x Seedtype x Time	8	9.429	0.726	0.668

Table 2. *Analysis of deviance (ANODEV) table on logit-transformed proportion of germinated seeds for seedtype, genotype and soil coverage.*

Source	df	Deviance	F	P
Seedtype	2	228.241	36.41	<0.001
Genotype	2	0.021	0.001	0.997
Soil Coverage	1	2000.492	639.23	<0.001
Seedtype x Genotype	4	7.181	0.57	0.685
Seedtype x Soil Coverage	2	244.569	39.07	<0.001
Genotype x Soil Coverage	2	0.486	0.08	0.926
Seedtype x Genotype x Soil Coverage	4	37.889	3.03	0.045

Figure 1.
Mean field germination for seedtype by time of exposure interaction. Dashed lines indicate point estimates for corresponding laboratory-stored seed treatments. (Error bars are ± s.e.).
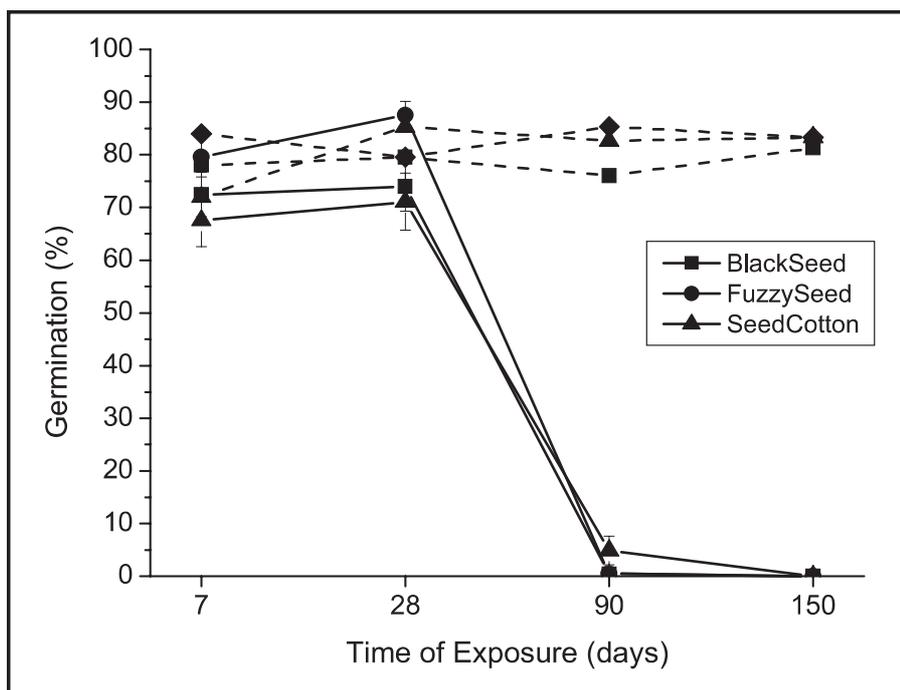

Figure 2.
Mean field germination for seedtype by genotype interaction. Hatched bars indicate point estimates for corresponding laboratory-stored seed treatments. (Error bars are ± s.e.).
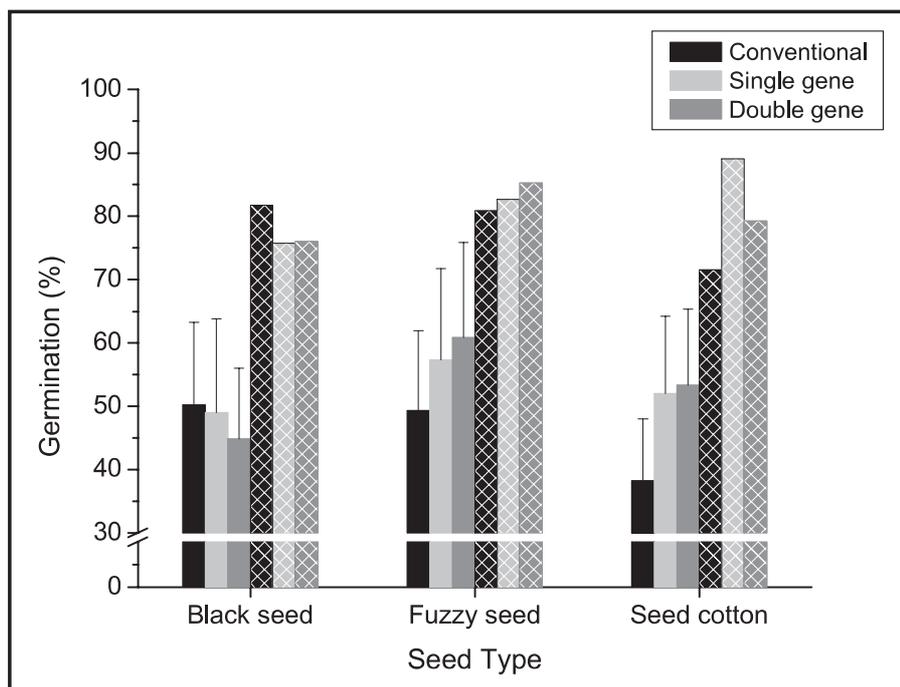
Figure 3.
Mean germination for seedtype by depth interaction (error bars are ± s.e.).
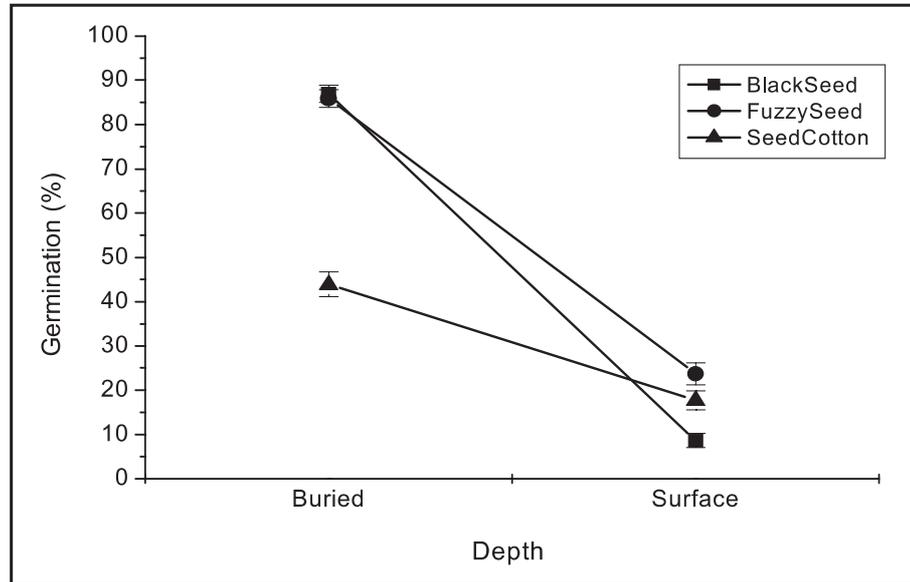

Figure 4.
Mean field germination for seedtype by genotype by depth interaction. Broken lines represent the surface treatments. (Error bars are ± s.e.).