

1224 Development of CLEARFIELD Cotton through Chemical Mutagenesis

Efrem Bechere (Corresponding Author)
Plant and Soil Sciences Department
Texas Tech University
Box 42122
Lubbock, TX 79409-2122
Phone: 806/767-9357
Fax: 806/742-0775
Email: efrem.bechere@ttu.edu

Dick L. Auld
Plant and Soil Sciences Department
Texas Tech University
Box 42122
Lubbock, TX 79409-2122
Phone: 806/742-5704
Fax: 806/742-0775
Email: dick.auld@ttu.edu

Peter A. Dotray
Texas Tech University, Texas Agricultural Experiment
Station, Texas Cooperative Extension
Lubbock, TX 79409-2122
Phone: 806/742-1634
Fax: 806/742-0775
Email: peter.dotray@ttu.edu

Lyndell V. Gilbert
Texas Agricultural Experiment Station
Lubbock, TX 79403
Phone: 806/742-1634
Fax: 806/746-6528
Email: LVGilbert@aq.tamu.edu

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ABBREVIATIONS: HVI (High Volume Index), GMO (Genetically Modified Organism), EMS (ethyl methanesulfonate)

Abstract

The induction of genes conferring herbicide resistance by mutagenesis could facilitate the use of imidazolinones as an alternative weed control system in upland cotton (*Gossypium hirsutum* L.). In 1997 and 1998, some High Plains cotton cultivars (Atlas, Explorer, Holland 338, Tejas, SC 9023, Sphinx and Rocket) were treated with 2.45% v/v EMS (ethyl methanesulfonate). The resulting mutants were then sprayed with imazethapyr and later on with imazamox with the objective of identifying tolerant mutants to this herbicide. Imazamox rates used were, 0, 87.5, 175, 350, and 700 g ai/ha. Four stable M₆-M₇ lines with good level of tolerance to imazamox were identified in 2004. These lines contained four genetic backgrounds – EM₄-3-1 (from Explorer), SCM₃-4-3 and SCM₃-7-3 (from SC 9023), and RM₃-8-1 (from Rocket). During 2005 and 2006, tolerant mutants with their original parents were planted in replicated trials at Lubbock and treated with the five imazamox rates. The log-logistic model was used to explain the dose-response relationship for lint yield. Excellent level of tolerance by these mutants to imazamox was observed. Imazamox doses, however, did not have a significant impact on fiber length, fiber strength and micronaire. The non-GMO cotton developed with this method is not a transgenic technology.

Key Words: Cotton, imazamox, imazethapyr, mutagenesis

The use of herbicides has revolutionized weed control in many crop production systems. Effective weed control is a prerequisite if high yields and quality are to be achieved. Current herbicide technology, including the use of glyphosate resistant crops, has improved weed control options but additional weed control options are still needed.

The developed non-GMO cotton mutants with tolerance to imidazolinone herbicides could provide growers with reliable, broad-spectrum weed control resulting in strong value-added marketable crop. CLEARFIELD[™] cotton is not a transgenic technology because it is developed through mutation and not gene transfer.

The induction of genes conferring resistance by mutagenesis could facilitate the use of imidazolinones as an alternative weed control system in upland cotton (*Gossypium hirsutum* L.). Imidazolinone herbicides include imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz, and imazaquin (Duke and Ragsdale, 2004).

Acetohydroxyacid synthase, also known as acetolactate synthase (ALS) is the first enzyme that catalyzes the biochemical synthesis of the branched chain amino acids – valine, leucine, and isoleucine (Singh, 1999). This enzyme is the site of action of the imidazolinone herbicides (Shaner et al., 1984). The imidazolinone herbicides inhibit the production of ALS and deprive the plant of the three essential amino acids. This herbicide target site is reported to have the highest incidence of developing resistance to herbicides (Shimizu et al., 2002). The first imidazolinone herbicides were available in the 1980s (Tan et al., 2005) and resistance was reported within 5 years. Point mutations resulting in Ala₁₂₂ substitutions in domain C (Wright et al., 1998; Jander et al., 2003) and Serine(Ser)₆₅₃ substitutions in domain E (Sathasivan et al., 1991; Lee et al., 1999; Jander et al., 2003) confer high resistance solely to imidazolinones (Pozniak et al., 2004).

Plants resistant to the imidazolinones have been successfully produced by seed, microspore, pollen and callus mutagenesis and somatic cell selections in maize (*Zea mays* L.) (Newhouse et al., 1991), *Arabidopsis thaliana* (L.) Heynh (Haughn and Somerville, 1986; Sathasivan et al., 1991; Mourad et al., 1993; Wright and Penner, 1998), canola (*Brassica napus* L.) (Swanson et al., 1989), cotton (Subramanian et al., 1990); Rajasekaran et al., 1996), soybean (*Glycine max* (L.) Merr) Sebastian et al., 1989), tobacco (*Nicotiana tabacum* L.) (Chaleff and Ray, 1984; Creason and Chaleff, 1988), wheat (*Triticum aestivum*) (Newhouse et al., 1992; Pozniak and Hucl, 2004), sunflower (*Helianthus annuus* L.), rice (*Oryza sativa* L.) (BASF Press Release, 29.08.2000) and alfalfa (*Medicago sativa*). These crops were commercialized as CLEARFIELD[™] crops from 1992 to present. Most of the mutations are due to single amino acid sequence changes that do not affect the enzyme function but easily induce herbicide resistance in plants where they occur. In nearly all cases, a single, partially dominant nuclear gene conferred resistance (Pozniak and Hucl, 2004). A single target-site mutation in the ALS gene may confer tolerance to ALS-inhibiting herbicide, so that it is technically possible to develop the imidazolinone tolerance trait in many crops (Tan et al., 2005).

The imidazolinone herbicides are absorbed by roots and foliage and translocated throughout the plant (both symplastically and apoplastically) accumulating in the meristems such as apical buds at root and shoot tips and axillary buds in leaf axis. Symptoms on cotton include red veins in leaves, shoot meristems cease growth, roots develop poorly and the secondary roots are shortened and all nearly the same length. Complete symptom development is very slow and requires two to three weeks.

Imidazolinone herbicides control a broad spectrum of grass and broadleaf weeds including cocklebur (*Xanthium strumarium*), velvetleaf (*Abutilon theophrastii*), ragweed (*Ambrosia artemisiifolia*), fall panicum (*Panicum dichotomiflorum*), foxtails (*Setaria faberi*), barnyardgrasses (*Echinochloa spp.*), nightshades (*Solanum sarrachoides*), annual morningglory (*Ipomoea spp.*), pigweed (*Amaranthus spp.*), nutsedge (*Cyperus spp.*) and lambsquarter (*Chenopodium album*) (Anderson et al., 1997). Imidazolinone tolerant crops may also prevent rotational crop injury. Cotton, which can be injured by residues of imidazolinone herbicides, commonly is rotated with peanut in North Carolina and across the Southeastern production region (York et al., 2000).

In addition to their broad-spectrum weed control and providing increased herbicide options for cotton growers, imidazolinone herbicides have other attractive characteristics. They can be applied pre plant, pre emergence, or post emergence. Post emergence options are especially important in no-till or reduced tillage systems or in fields with variable weed pressures. They are environmentally attractive because they possess high biological potency, making them very effective at low application rates (Newhouse et al., 1991). Since branched chain amino acids biosynthesis does not occur in animals, the imidazolinones are relatively non-toxic to animals (Pozniak and Hucl, 2004). We believe the work on developing imidazolinone tolerant lines in cotton has involved only a GMO approach. Anderson et al. (1997) isolated genes encoding acetohydroxyacid synthase from cotton, engineered to express imidazolinone resistant form of the enzyme and reintroduced this into cotton utilizing an *Agrobacterium* mediated transformation system.

Imidazolinone tolerant crop species are being marketed for use with imazethapyr or imazamox. Imazamox is a new member of the imidazolinone family of herbicides and has a few advantages over imazethapyr. It exhibits better control of grasses and common lambsquarters and also has a soil life about one-half of imazethapyr (Webster and Kapusta, 1997; University of California, 2002). The active ingredient rate for imazamox is 0.027 to 0.526 kg/ha and is half of that of imazethapyr which is 0.526 to 0.105 kg/ha.(University of California, 2002).

Nonlinear regression using the log-logistic model is the method that is currently best suited to explain herbicide dose-response relationships (Seefeldt et al., 1995).

Objective

The objective of this research was to determine imazamox tolerance of some chemically mutated upland cotton cultivars.

Materials and Methods

In the 1997 crop season, seeds of six High Plains cotton cultivars (Atlas, Explorer, Holland 338, Tejas, SC 9023 and Sphinx) were treated with 2.45 % v/v EMS (ethyl methanesulfonate). In 1998, two additional populations (Rocket and Explorer) were also treated with 2.45 % v/v EMS. Starting in 1997, the M_1 plants were grown to produce M_2 seeds at the field at Lubbock. During 1998 and 1999 growing seasons, 14,400 M_2 plants (from the first six populations) and 38,400 M_2/M_3 plants (from all the eight populations),

respectively, were treated with imazethapyr at the rate of 87.5 g a.i./ha. For the 2000 growing season, 113 M₃/M₄ lines selected from the previous year's populations, were planted in the field at Lubbock and sprayed with imazethapyr at the rate mentioned above. One hundred and six tolerant lines were selected and planted as M₄/M₅ in 2001 in the field at Lubbock. Eleven imazethapyr tolerant M₅/M₆ lines were then promoted to 2002, planted in the greenhouse and treated with 87.5 and 350 g ai/ha imazamox (i.e 2X and 8X the recommended rate in soybean). Eighteen M₆/M₇ lines that showed good levels of tolerance to imazamox and three check varieties were planted in the field in 2004 and again treated with 87.5 and 350 g ai/ha imazamox. The 2004 field trial identified four stable mutants in three different genetic backgrounds (EM₄-3-1, SCM₃-4-3, SCM₃-7-3, and RM₃-8-1) that showed very good tolerance levels to imazamox. These four mutants along with the original non-mutated commercial parents were planted in the field at Lubbock during 2005 and 2006 in a randomized block design in three replicates and treated with 0, 87.5, 175, 350, and 700 g ai/ha imazamox. Plants were sprayed at 30 days post emergence at the 4-6 leaf stages.

Nonlinear regression using the log-logistic model described by Seefeldt et al. (1995) was used to explain the imazamox dose-response relationships for lint yield. The dose-response curves were plotted using a logarithmic (geometric) scale. Imazamox doses (independent variable) were plotted against lint yield (dependent variable). The dose-response relationships are nonlinear and a nonlinear regression routine, PROC NLIN in the SAS package (SAS Institute Inc., SAS Circle, Carry, NC) was used to estimate the parameters of the log-logistic response curves. This procedure required initial estimates of starting values of the parameters C, D, b, and I₅₀ where C=lower limit,

D=upper limit, b=slope and I_{50} =dose giving 50% response. These were obtained from visual evaluations of the data plot generated by PROC PLOT in the SAS software (SAS Institute Inc.). The mean responses for untreated entries and for entries receiving high doses of imazamox were used as starting values of D and C, respectively. To estimate values of b, b=2 and b=3 were used as starting points for susceptible and tolerant entries, respectively. These values were used as a basis for obtaining a revised set of parameters that make the model predict the fit of the data better through PROC NLIN. These revised set of parameters were used to plot the log-logistic curves using the SigmaPlot program (SigmaPlot 2000, SPSS Inc., Chicago, IL).

Results and Discussion

Symptoms

Cotton symptoms following an imazamox treatment included red veins on leaves (Figure 1), stunted shoot meristems, roots developed poorly and the secondary roots were shortened and all nearly the same length (Figure 2). Complete symptom development was very slow. The symptoms were more apparent at the higher rates of imazamox. Even though chlorosis was observed at the earlier stage of application, the tolerant mutants recovered and developed into normal plants while the check varieties remained yellow and eventually died (Figures 3 and 4).

Lint Yield

A combined analysis of variance for lint yield across 2005 and 2006 indicated differences between the two years ($F=23.8^{**}$, $P=0.0001$), therefore, data from the two years were analyzed separately. Within each dose, however, no differences were observed between the tolerant mutants and also between the susceptible cultivars. Lint yield data from each dose across the tolerant mutants were combined for the log-logistic analysis. The same procedure was followed for the susceptible cultivars.

An analysis of variance using the PROC GLM procedure for both the 2005 and 2006 lint yield data showed differences between the different doses ($F=41.7^{**}$, $P=0.0001$ for 2005 and $F=42.8^{**}$, $P=0.0001$ for 2006). Differences were also observed between the entries ($F=369^{**}$, $P=0.0001$ for 2005 and $F=224^{**}$, $P=0.0001$ for 2006). Dose by entry interaction was only significant in 2005 ($F=7.09^{**}$, $P=0.0001$). The models in both years explained the data well ($F=62.8^{**}$ and $F=42.8^{**}$ in 2005 and 2006, respectively). Eighty-six percent and 81 percent of the variations were explained by the models in 2005 and 2006, respectively. No block effects were observed for both years and were therefore excluded from the analysis.

The log-logistic curves for the tolerant mutants and the susceptible check cultivars are given in Figure 5. As the imazamox rates increased, the lint yields of the tolerant mutants were only slightly decreased whereas the yields of the susceptible check cultivars decreased significantly. The I_{50} , which is the imazamox dose that gave 50% lint yield response, was 59 in 2005 and 127 in 2006 for the susceptible cultivars. For the tolerant mutant cultivars, these values were 1408 and 1475 for 2005 and 2006, respectively. These means that an imazamox rate of 1408-1475 kg ai/ha has to be applied on these

tolerant mutants before there is a 50% lint yield reduction. This corresponds to about 33-fold increase in the current labeled rate for imazamox use in soybean. The results demonstrated that these mutants have excellent tolerance to imazamox.

Fiber Quality

HVI fiber quality for the resistant mutants and the check cultivars was carried out at the Texas Tech University International Textile Center at Lubbock, TX. The GLM Procedure indicated no significant differences between the imazamox doses for fiber length in 2005 ($F=2.34^{NS}$, $P=0.0609$) and 2006 ($F=0.85^{NS}$, $P=0.4970$). Fiber strength differences were not significant in 2005 ($F=1.61^{NS}$, $P=0.1792$) but significant in 2006 ($F=5.34^{**}$, $P=0.0006$). Imazamox dose by entry interactions were not significant in both years. The model only described 10-18% and 12-36% of the variations in fiber length and fiber strength in 2005 and 2006, respectively. No attempts were made to fit these data to the log-logistic model.

Summary and Conclusion

Four imazamox tolerant cotton mutants and three check commercial cultivars have been repeatedly tested in the greenhouse and field conditions with 0, 87.5, 175, 350, and 700 g ai/h of imazamox. Lint yield data in 2005 and 2006 have confirmed that these mutants are tolerant to imazamox. Even at 16 times the normal recommended rate of imazamox (in soybean), these mutants showed a considerable level of tolerance to imazamox. Fiber quality data indicated that imazamox doses do not significantly affect fiber length, fiber strength and micronaire readings.

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- Figure 3** **Field response of a cotton mutant and a commercial cultivar to imazamox at 350 g ai/ha.**
- Figure 4** **Effect of imazamox (350 g ai/ha) on a commercial cultivar and its resistant mutant at maturity.**
- Figure 5** **Log-logistic analysis (lint yield) of imazamox tolerant mutants and susceptible cultivars grown at Lubbock, TX during 2005 and 2006.**

Figure 1



Figure 2.



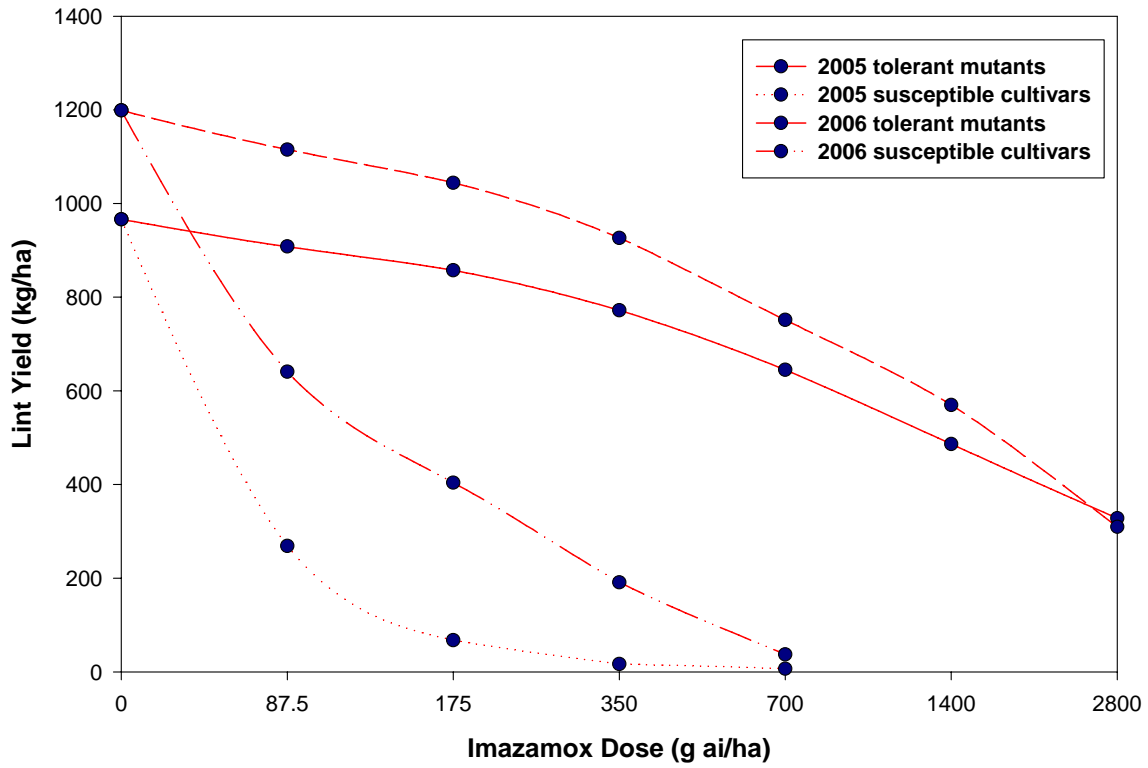
Figure 3.



Figure 4.



Figure 5.



The mathematical expression relating the response y to the dose x is:

$$y = f(x) = C + \frac{D - C}{1 + (x/I_{50})^b}$$

$$= C + \frac{D - C}{1 + \exp[b(\log(x) - \log(I_{50}))]}$$

Where C = lower limit, D = upper limit, b = slope, and I_{50} = dose giving 50% response.