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N-HIBIT® SEED TREATMENT AND PROACT®: HARP-N-TEKTM PRODUCTS FOR USE IN COTTON PRODUCTION

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Abstract

N-Hibit™ and **ProAct™** are new **Harp-N-Tek™** products from Eden Bioscience that show promise for enhancing cotton production. N-HIBIT contains 3% harpin$_{EA}$ protein and is formulated as a seed treatment. ProAct contains 1% harpin$_{\alpha\beta}$ protein as the active ingredient and is applied as a foliar spray. Harp-N-Tek products are based on harpin proteins, which activate the innate ability of a cotton plant to protect itself through growth and stress-defense responses. These responses enhance cotton's overall plant health and yield potential by improving vigor, stamina, nutrient uptake, and reproductive growth and by initiating natural self-defense mechanisms. In 2004, 20 large scale Experimental Use Permit (EUP) trials and four replicated, small plot trials with ProAct were conducted by independent crop consultants and agricultural scientists in AL, AR, GA, LA, MS, and NC. ProAct at one ounce per acre increased lint yield, number of open bolls, and pounds of lint per 1,000 open bolls. The optimal timing for ProAct is as a tank mix with glyphosate applied during the two to four leaf stages of cotton. At the 2005 Beltwide Cotton Conference, research findings with N-Hibit will be covered in much greater detail at two presentations in the Cotton Disease Council conference (Kirkpatrick et al. 2005, Lawrence et al. 2005). N-Hibit offers a new technology to turn on plant defenses from the **inside-out** at planting and consequently reduces numbers of nematode eggs. Further, N-Hibit complements other nematode management tools. Evaluations of N-Hibit and ProAct on cotton will continue in the 2005 season.

Introduction

N-Hibit™ and ProAct™ are products based on Harp-N-Tek™, which is a proprietary harpin protein technology from Eden Bioscience. The first harpin protein-based product introduced into production agriculture was Messenger® (harpin$_{EA}$). N-Hibit™ seed treatment and Mighty-Plant™ are also registered for use. ProAct, harpin$_{\alpha\beta}$, is currently in the EPA registration process. Both N-Hibit and ProAct are expected to be commercially available for the 2005 cotton production season.

Harpin Proteins

Harpin proteins are natural compounds produced by disease-causing bacteria that attack plants (Wei et al., 1992). In the early 1990s, Cornell scientists discovered that fire blight, *Erwinia amylovora*, releases a protein (harpin$_{EA}$) while attacking apple trees (Wei et al. 1992). Harpins are acidic, heat stable, glycine-rich, extracellular (cell-envelope-associated-protein) with a molecular weight of ~40 kilodaltons and consists of ~400 amino acid residues with no cystiene. Harpin protein was the first broad-spectrum elicitor of the hypersensitive response to be discovered (Wei et al. 1992).

Harpin proteins are not part of the destructive disease complex; instead, they serve a beneficial purpose of alerting plants that they are under attack. Plants are naturally equipped with early warning receptor molecules that detect harpin proteins. When a Harp-N-Tek product is applied to cotton, the harpin protein active ingredient binds to the harpin receptors on the plant. Receptors respond to harpin as if it were a pathogen. The plant reacts by sending a systemic signal thereby initiating a sequence of physiological and biochemical reactions which ultimately activate the plant's intrinsic ability to protect itself through both growth and stress-defense responses. This warning signal initiates an "**inside-out plant response**" by turning on the plant's own natural capabilities.

The induced systemic response that is activated by harpins has been associated with enhanced resistance in plants to pathogens and certain other pests (Wei & Beer, 1996). **Plant Health Regulator** products that contain harpins, such as N-Hibit™, Messenger® STS and ProAct™ , have been developed and commercialized in response to these findings. Harp-N-Tek™ is the title for technologies originating from harpin proteins, and all Harp-N-Tek technologies initiate an "**inside-out plant response**" by turning on the plant's own natural capabilities.

Plant defenses have been investigated and reviewed in great detail (Agrawal et al. 1999, Agrios 1997, Keen et al. 2001). Harpin-induced defense effects include hypersensitive response, ion exchange and oxidative burst which induces production of active oxygen and K/Cl effluxes and Ca/H influxes, induction of salicylic acid accumulation locally and systemically, activation of multiple plant defense pathways including salicylic acid pathways, jasmonic acid pathways, phenylalanine ammonia-lyase (PAL) mediated pathways, as well as novel gene induction and pathways (Wei, personal communication, 2004).
Harpin-induce plant growth effect can include increased ion exchange reaction, elevated protein, sugar, starch transportation, increased nutrient uptake, increased net photosynthesis, and physiological changes associated with transpiration and respiration (Wei, personal communication, 2004). Through numerous laboratory, greenhouse, and field trials, some of the benefits of associated with the use of Harp-N-Tek based products have been found to include increased root biomass, improved nutrient utilization, nematode egg suppression, and increased yield and quality

By separating the harmless signal inducing harpin proteins from the harmful disease-causing bacteria, Eden Bioscience's Harp-N-Tek products trigger beneficial responses that protect plants, help plants grow through stress, and enhance the overall level of plant health. Harpins have no direct effect on pests; instead, harpins turn on natural growth and stress mechanisms within plants. Activation of natural stress-defense and growth responses in plants can initiate increased plant growth, stamina, and vigor, improve overall plant health, and can lead to improved output quality, increased marketable yields, and enhanced shelf-life. Furthermore, nutrient utilization by crops has been positively affected by harpins. Other attributes of Harp-N-Tek products include low use rates, rapid degradation in the environment, little or no dietary exposure, negligible toxicity, ease of application, and mass production using simple, environmentally friendly, and cost-effective water-based fermentation technology. Harp-N-Tek products are formulated with low risk inert ingredients similar to those used in Messenger.

In addition to topical use of harpins for seed treatment and foliar spray, the gene for $harpin_{EA}$, which is the active ingredient in Messenger, had been introduced into cotton to create transgenic harpin cotton. Results from the first evaluation of transgenic expression of $harpin_{EA}$ against RKN are reported at the 2005 Beltwide Cotton Conference (Kirkpatrick et al. 2005).

Harpins have a very favorable safety profile. Acute toxicity tests document that harpin proteins are virtually non-toxic to mammals. Similarly, harpin proteins are classified as practically non-toxic to all other species tested (bobwhite quail, honeybee, plants, rainbow trout, *Daphnia magna*, and algae). Harpin protein is not persistent and does not accumulate in the environment. Currently registered harpin protein-based products are classified as a Toxicity Category IV pesticide (EPA signal word "Caution" on label is optional). Minimal protective clothing is required to mix and apply safely, and the restricted entry interval (REI) is 4-hours which is the minimum required by the EPA. Harpin is exempt from the requirement of a tolerance for all food crops.

N-Hibit Seed Treatment

N-Hibit (EBC-151 ST, EBC-152, EBC-583) contains 3% $harpin_{EA}$ protein and is formulated as a seed treatment. Testing in replicated University cotton trials has demonstrated positive effects against nematodes. N-Hibit and other Harp-N-Tek products have been shown to have an adverse effect on nematode populations in that nematodes produce fewer eggs per root weight. Nematodes caused an estimated 4.24% reduction in cotton yields in 1999 or a loss of 727,215 bales (Blasingame & Patel 2000). The key nematode species contributing to yield losses are root-knot nematode, *Meliodogyne incognita*, and reniform nematode, *Rotylenchulus reniformis* (Mueller 2000). The primary objective of research with N-Hibit was to determine if use of N-Hibit as a seed treatment on cotton influences the reproduction of *R. reniformis* or *M. incognita* on cotton.

ProAct Foliar Treatment

ProAct contains 1% $harpin_{\alpha\beta}$ as the active ingredient and is applied as a foliar spray. $Harpin_{\alpha\beta}$ is comprised of harpin protein fragments derived from several native harpin proteins found in naturally occurring. Previous tests from both green house and field trials indicate that ProAct has higher activity than $harpin_{EA}$. Favorable yield results were obtained in previous evaluations of ProAct in cotton, corn, rice, and other crops. With ProAct, Eden Bioscience chose to investigate application during or before a known stress event. Post-emerge herbicides were selected because plant tolerance to herbicides tends to come from a plant's ability to detoxify the herbicide and continue to grow, thereby requiring energy. The most widely used post-emerge herbicide is glyphosate, used on

Roundup Ready® crops. Field data from replicated plots and commercial demonstrations on Roundup Ready® corn and cotton consistently documented that adding ProAct to a glyphosate application provides yield increases in the 6% to 12% range, depending on crop and conditions. Intensive testing of ProAct in cotton continued in 2004. In 2004, the Environmental Protection Agency (EPA) granted an Experimental Use Permit (EUP) for EBC-351 (ProAct). Consequently, commercial evaluations of ProAct by cotton consultants were initiated in demonstration trials this season. Similarly, replicated, small plot trials were conducted with EBC-351.
The primary objective of research with ProAct on cotton was to evaluate the influence of rate and timing on ProAct performance in cotton, primarily in commercial production conditions. Findings from that research are reported.

Materials and Methods

ProAct Large Block Demonstration Trial Methodology

Design. During 2004, ground applications of EBC-351 (ProAct) were extensively evaluated in commercial cotton fields. ProAct EUP cooperators were independent agricultural crop consultants from AL, AR, GA, LA, MS, and NC (Table 1). Twenty-one trials were initiated. Due to weather-related issues, one trial was not completed, and another trial was treated during mid-bloom. Each cooperator chose the farmers and fields for the trials. Each site was planted with a locally adapted variety that was tolerant to glyphosate and expressed *Bacillus thuringiensis* protein. Further, each site had a reasonably uniform soil profile and was planted within a commercially acceptable planting window. The field selected for each trial was partitioned in three to five similar blocks depending on the number of test treatments. At some locations, treatments were replicated from two up to four times. Due to EUP acreage restrictions, plot size for each treatment was typically limited to 8 to 10 acres. All blocks were treated identically except for the application of ProAct. Cooperators were asked to clearly mark the limits of each plot, using flags, stakes or whatever is available and durable as well as to document GPS coordinates. Plant growth inputs, insects, mites, and weeds were managed according to locally accepted practices, and all plots within a given trial were treated identically. Weather and production inputs such fertility program, pesticide use, and defoliants were documented in a *Site Description Form* that was provided to each consultant.

Treatments and Application. Treatments consisted of ProAct applied at two timings: two to four leaf stage or eight leaf stage. ProAct was tested at rates of 1 and 2 ounces per acre. Each trial included an untreated control that was not treated with ProAct. Cooperators were requested to apply ProAct treatments with properly calibrated ground equipment preferably equipped with a shielded spray boom to minimize spray drift, to avoid spraying on a windy day when spray applications are prone to drift, and to make sprays on a day and at a time when the plants are actively growing. Water was used as the carrier, and treatments were applied at a finished spray volume of 10 to 20 gallons per acre. Cooperators applied ProAct as stand alone applications or in tank mixtures with other products. A typical application at the two to four leaf stage consisted of ProAct and glyphosate.

Plant Stand and Early Growth Observations. Beginning one week after treatment, plots were scouted at least weekly and assessed for visual differences in plant establishment and early growth. Scouting continued through at least early bloom, and observations were reported in an *Early Season Observation Form*.

Pre-Harvest Boll Measurements. After defoliation, numbers of open, green or immature, hard lock, and rotten bolls from each plot were recorded for two adjacent seven-row ft samples. Samples were collected from areas in each plot with uniform growth, and sampling within 100 feet of the field edges and from atypical areas and plants was avoided. The procedure was repeated for at least four separate sample areas in each treatment and measurements were recorded in a *Cotton Boll Measurements Form*.

Cotton Yield. Plots were harvested, and yield as pounds of seed cotton per acre were recorded for each plot. Fiber samples were ginned, and percent turnout was calculated. Seed cotton yields were converted to pounds of lint per acre.

ProAct Replicated, Small Plot Field Trial Methodology

Design. The experimental design of each trial was completely randomized. If blocking factors were identified, then the design was adjusted to randomized complete block. Treatments were replicated seven or eight times. Plots contained four treated rows of cotton (generally 12.6 ft wide, 50 ft long) planted on 36 to 38 inch row spacing. Each plot included a buffer of 10 ft between blocks and at least 2 rows between adjacent plots. Each site was planted with

a locally adapted variety that was tolerant to glyphosate and expressed *Bacillus thuringiensis* protein. Further, the selected site had a reasonably uniform soil profile and was planted within commercially acceptable planting window. At planting, aldicarb was applied at 3.5 lb per acre in-furrow. Plant growth inputs, insects, mites, and weeds were managed according to locally accepted practices, and all plots within each trial were treated identically.

Treatments and Application. Independent, private agricultural scientists conducted all replicated trials. Four trials were completed with cooperators located in AR, LA, and NC. Treatments consisted of EBC-351 (ProAct) applied at two timings: two to four leaf stage or eight leaf stage. Several rates were tested, particularly 1 and 2 ounces ProAct per acre. Each trial included an untreated control that was not treated with ProAct. Cooperators were requested to apply ProAct treatments with properly calibrated ground equipment utilizing a four-row shielded spray boom with two spray nozzles per row and to apply each plot as single pass. Water was used as the carrier, and treatments were applied at a finished spray volume of 10 gal/acre. Cooperators applied ProAct treatments as stand alone applications without adjuvants or pesticides. Spray equipment was carefully rinsed prior to each application. Sprays were made on a day and at a time when the plants are actively growing.

Field Observations. Prior to or at planting a composite soil sample was collected and analyzed for OM, micronutrients, macronutrients, soil pH, calculated CEC, and percent cation saturation. As appropriate, phytotoxicity ratings were taken and expressed as percent damaged or stunted. Because no adverse effects were observed, results will not be further discussed.

Maturity Measurements. From each plot, 5 consecutive plants from each of the two center rows were evaluated. If a plant was missing a terminal, the cooperator skipped the atypical plant and moved on to the next plant until 5 plants had been sampled. Sampling was not done near plot edges. Pool measurements for 10 plants, report as mean. At approximately nodes above white flower (NAWF) 5 or 6, NAWF was assessed on each plant by counting nodes above first position white flower to the unfurled leaf. Sample plants with first position white flowers. NACB: Record number of mainstem nodes between uppermost first-position cracked and last harvestable boll on each plant were assessed for nodes above cracked boll (NACB) at approximately NACB = 7.

Pre-Harvest Measurements. At harvest or within two weeks of harvest, plant height for 5 representative plants from center area of each plot was measured. Numbers of plants and open, green, hard lock, and rotten bolls from each plot were recorded for two seven-row ft samples from both center rows. Data were pooled to numbers per 14 row ft.

Cotton Yield. The center two rows of each plot were harvested with a cotton picker, and harvested seed cotton from each plot was weighed and converted to a per acre yield estimate. A small sample from each plot was labeled and shipped to a private laboratory for ginning. During the ginning process, pre-ginning weight, seed weight, and lint weight were recorded and percent turnout was calculated. Seed cotton yields were converted to lbs of lint per acre.

Replicated Trial Data Analysis. Data were subjected to an analysis of variance (ANOVA) and Duncan's New MRT (p=0.10, protected) means separation test (Duncan 1955, Cochran & Cox 1957). Significance is reported at P=0.05 for analyses unless otherwise indicated. In many ANOVAs of the field trial data, results were not significant at the P=0.05 level. Consequently, most of the results and discussion will focus on trends and patterns.

Pooled Data Analysis. Data from large block demonstration trials and replicated, small plot field trials were pooled for analysis. Because the ProAct and comparison treatments were not always applied at each site, sample size reported may differ from the numbers of paired comparisons and numbers of data points. For each measurement, median values were calculated for combinations of treatment timing and rate. Percent positive above control value was calculated for each value, and results were tabulated. Chi-squared tests were used to test the hypothesis that the outcomes, as number of positive and negative percent differences, would occur in a 1:1 ratio (Steel & Torrie 1980). This report focuses on results with ProAct at one ounce per acre.

N-Hibit Replicated Trial Methodology

Over the past several years, University plant pathologists and agronomists have completed a series of greenhouse and growth chamber trials that included evaluations of N-Hibit seed treatment. Results are reported in another presentation at the 2005 Beltwide Cotton Conference (Kirkpatrick et al. 2005).

Results and Discussion

Findings with ProAct. ProAct at one ounce per acre provided a substantial increase in lint yield, numbers of open bolls, and pounds of lint per 1,000 open bolls (Table 2). Cotton treated with ProAct mixed with glyphosate and after glyphosate yielded 11% and 9%, respectively, above the untreated control. Of the numerous data pairings for yield, over 80% were positive for ProAct yielding above the control; and based on Chi-Squared analysis, and these findings were significant. Counts of open bolls documented one likely source for the yield increase. ProAct treated cotton had 4% to 7% more open bolls compared with the untreated control, and 88% to 100% of these pairings were positive, another significant finding. Furthermore, calculating boll weights as pounds of lint per 1,000 cotton bolls revealed that plants treated with ProAct had bolls that were 3% to 5% heavier than the control.

In replicated trials, analysis of plant growth and maturity measurements revealed minor differences among treatments for NAWF, NACB, and plant height; however, numbers of open bolls and lint yields with ProAct were consistently and considerably greater than the untreated control (Table 3).

Findings with N-Hibit. At the 2005 Beltwide Cotton Conference, research findings with N-Hibit will be covered in much greater detail at another presentation in the Cotton Disease Council conference, and this related paper will be published in the 2005 Proceedings from the 2005 Beltwide Cotton Conference.

> MESSENGER®, HARPIN SEED TREATMENT, AND HARPIN$_{EA}$ GENE TRANSGENIC COTTON REDUCE REPRODUCTION BY ROOT KNOT AND RENIFORM NEMATODES, T. L. Kirkpatrick, University of Arkansas Southwest Research & Extension Center, N. M. French, II, Eden Bioscience Corporation, J. R. Rich, University of Florida North Florida Research & Education Center, Z-M. Wei, Eden Bioscience Corporation.

Positive effects have been observed in greenhouse and growth chamber experiments with N-Hibit (Kirkpatrick et al. 2005). Cotton plants treated with N-Hibit have demonstrated improved plant growth, such as nodes per plant and root weights, and decreased fecundity (~55% lower), measured as numbers of nematode eggs per root weight.

Conclusions

In summary, Harp-N-Tek products N-Hibit and ProAct are new materials with unique activity that show promise for enhancing cotton production. Harp-N-Tek products are based on harpin proteins, which activate cotton's ability to protect itself through growth and stress-defense responses. These responses enhance cotton's overall plant health and yield potential by improving vigor, stamina, nutrient uptake, and reproductive growth and by initiating natural self-defense mechanisms.

Based on field trials conducted during the 2004 season, ProAct timing trials documented significant beneficial effects on cotton yield. ProAct increased lint yield, numbers of open bolls, and weight of lint per boll. Current results demonstrate that treatment with ProAct can improve the productivity of cotton. The optimal timing for ProAct is as a tank mix with glyphosate applied during the two to four leaf stages of cotton. Evaluations of ProAct trials on cotton will continue in the 2005 season.

N-Hibit offers a new technology to turn on plant defenses from the **inside-out** at planting and consequently reduces numbers of nematode eggs. Further, N-Hibit complements other nematode management tools. Research is underway to further investigate the influence of N-Hibit and other Harp-N-Tek products on nematodes infesting cotton. Field evaluations will be initiated in 2005.

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Table 1. Cooperators for large block EUP demonstration trials and replicated, small plot field trials with ProAct, 2004.

Independent Consultant or Agricultural Scientist	Protocol Number	Trial Number	State
Paul Clark	CT-04-04	204029	AL
Dr. Richard Davis	CT-04-04	204030	AL
Chuck Farr	CT-03-04	204027	AR
Dr. Merritt Holman	CT-04-04	204031	AR
Eddy Cates	CT-04-04	204032	AR
Danny Moore	CT-05-04	204037	AR
Dr. Charlie Guy*	CT-02-04*	204019	AR
Jay Holder	CT-03-04	204028	GA
John Beasley	CT-04-04	204033	GA
Dr. Don Hays	CT-04-04	204034	GA
John Beasley	CT-05-04	204038	GA
Kevin Cotton	CT-05-04	204039	GA
Roger Carter	CT-03-04	204025	LA
Dr. Grady Coburn	CT-03-04	Too wet	LA
John Lee Godley*	CT-02-04*	204020	LA
John Lee Godley*	CT-02-04*	204021	LA
Tucker Miller	CT-03-04	204023	MS
Jeff North	CT-03-04	204024	MS
Billy Bryant	CT-04-04	204035	MS
Joe Townsend	CT-05-04	204040	MS
Dr. Winston Earnhart	CT-05-04	204041	MS
David Dubard	CT-05-04	204089	MS
Bill Peele	CT-04-04	204036	NC
Billy McLawhorn	CT-05-04	204042	NC
Paul Garvey*	CT-02-04*	204022	NC
Roger Carter	CT-03-04	204025	LA

* indicates replicated, small plot trial with 8 replications per treatment.

Table 2. Influence of ProAct on cotton lint yield, open bolls, and bolls weights as percent increase above the untreated control across all tested locations, 2004.

Plant Measurement	Applied with Glyphosate (2-4 leaf cotton stage)				Applied after Glyphosate (8-leaf cotton stage)			
	Median % Difference from Control	% Positive	N	X^2	Median % Diffference from Control	% Positive	N	X^2
Lint Yield (lbs/acre)	11.0%	80%	15	5.4*	9.0%	88%	16	9*
Open Bolls (per 14 row feet)	4.4%	88%	14	7.1*	7.3%	100%	15	15*
Lbs. of Lint per 1,000 open bolls	3.4%	71%	14	2.6	5.1%	60%	15	9.0

*significant difference with Chi-Square test ($P<0.05$).

Table 3. Influence of ProAct at 1 oz per acre on nodes above white flower, nodes above cracked boll, and plant height across replicated, small plot trials, 2004.

Treatment	Field Trial Number and State				Median	% Difference from Control
	204019-AR	204020-LA	204021-LA	204022-NC		
NAWF (Nodes Above White Flower)						
Untreated Control	6.4	5.2	5.3	6.3	5.8	n.a.
ProAct Applied with Glyphosate	6.2	5.2	5.4	5.9*	5.7	-3%
ProAct Applied after Glyphosate	6.2	5.4	5.2	6.0*	5.7	-2%
NACB (Nodes Above Cracked Boll)						
Untreated Control	7.2	7.2	7.4	5.7	7.2	n.a.
ProAct Applied with Glyphosate	7.2	7.4	7.3	5.6	7.3	1%
ProAct Applied after Glyphosate	7.1	7.3	7.3	5.6	7.2	0%
PLANT HEIGHT (inches per plant)						
Untreated Control	35.7	31.0	37.5	38.7	34.0	n.a.
ProAct Applied with Glyphosate	37.6*	31.9	38.1	40.5	35.8	5%
ProAct Applied after Glyphosate	36.7	31.8	38.3	39.8	35.3	4%
OPEN BOLLS (number per 14 row feet)						
Untreated Control	320	198	225	211	218	n.a.
ProAct Applied with Glyphosate	329	208	233*	257	245	12%
ProAct Applied after Glyphosate	321	204	238*	226	232	6%
LINT YIELD (pounds per acre)						
Untreated Control	1,283	746	919	968	943	n.a.
ProAct Applied with Glyphosate	1,377	1,006*	1,091*	1,196	1,143	21%
ProAct Applied after Glyphosate	1,398	1,008*	1,095*	1,147	1,121	18%

* Indicates value significantly difference from untreated control, Duncan's New MRT (p=0.10, protected).