SOFTER SHOCK

Case study - Frostban

APPLICATION OF GMOS ON CROPS TO PREVENT FROST DAMAGES

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Summary

Everlasting symbol of the clash between Genetically Modified Organisms and modern society, the Frostban case is very important for us to correctly understand.
This product; developed by the company Advanced Genetic Sciences (AGS), has induced the release of GMOs in the environment under consequent controversies in the late 80's.
Let's face it, Softer Shock can be considered as a twin of Frostban, because it aims at treating frost injuries on plants by using GMOs
So Frostban would be the elder twin of Softer Shock? Can we really compare both projects?

This report aims at giving an insight on what is Frostban precisely, and draw a comparison between it and Softer Shock. Can "Softer Shock" have a double meaning after all?

INTRODUCTION : Frost injury in North America and AGS

Advanced Genetic Sciences (AGS) was a company founded in 1980 by Daniel D. Adams (Protein sciences corporation, 2017), later acquired by DNA Plant Technology in 1986 (the fusion was done in 1987).
This company is known for its development of Frostban more than any other projects.

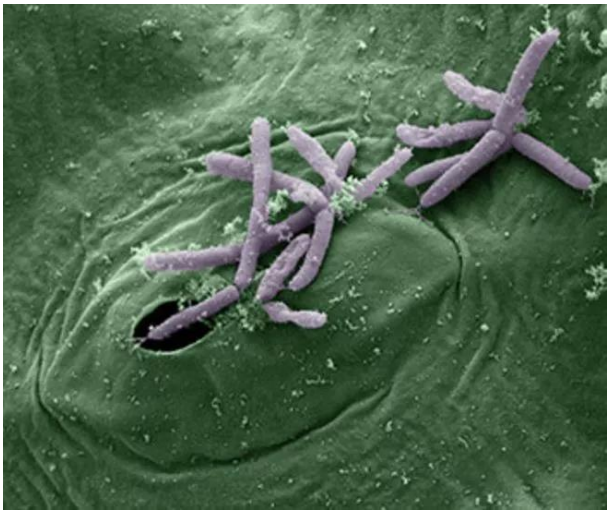
Not authorised to commercialise the product (we will try to find out why in this report), the company had hence a short lifespan at a time where GMOs were already causing many concerns and clash with society.

Why did AGS want to develop such product? They actually had the same objectives as us, aquired by analysing the damages caused at the time by frost to US and Canadian crops.

Indeed, in 1974, 1979 and 1982, cost of damages caused by frost injuries to spring grains, corn, oilseeds, and vegetables in the Canadian province of Saskatchewan was reported to range from 11 to 13 millions of dollars (13,4 millions for the Saskatchewan cereals alone in 1982) (Margaritis et al., 1991).

As for the USA, a 1975 report from the MIT has evaluated the total cost of frost injuries to one billion dollars each year (White 1975).
There was hence a place to take for companies that would offer alternative treatment to costly existing ones, such as heaters, water aspersion… and Fish AntiFreeze Glycoproteins (Margaritis et al., 1991).

And then there was this interesting opening given by independent researchers in Wyoming and Wiconsin, identifying certain bacteria, like *Pseudomonas syringae*, as being an ice nucleator hence potentially triggering frost injuries (Skirvin et al., 2000).



Pseudomonas Syringae, one of the first organism to be suspected of ice nucleation

Research after research, proteins causing the ice-nucleation activity were identified and named Ice-Nucleation Proteins (INPs), and AGS began to develop Frostban based on this molecular principle (Margaritis et al., 1991). The next part will briefly describe the product composition and logic.

I. Molecular and biological basis of Frostban

Microorganism spray on leaves to protect from frost. Seems rather familiar, right?

At this time consideration for the plant microbiota and metagenomics were not as developed as today (or simply didn't exist), so the organisms chosen for Frostban were *Pseudomonas syringae (strain RGP 36R2)*, and *Pseudomonas fluorescens (strain GJP 17BR2),* two *Pseudomonas* species occurring on plants and soil, that nucleate ice (Supkoff et al., 1987).

In this case study, we are not going to focus on the ice formation cycles and the damages caused by frost to crops, you can find such information in the report "Compound choice" on our wiki.

Rather we are first going to examine the organisms.
First, both strains selected were resistant to an antibiotic called rifampicin (Supkoff et al., 1987).
Note that such resistances are found in the wild and this is very likely that AGS selected their strains on these properties and did not rely on the use of vector DNA. It has been furthermore shown that resistance to rifampicin, that binds to RNA polymerase, is acquired by mutation of the RNA polymerase gene that changes the protein structure and make it inaccessible to the antibiotic (Hall et al., 2011).

The resistance hence is very likely to be on the chromosomal DNA rather than on a plasmid.
But of course the main point here is the genetic modification that both *P.syringae* and *P.fluorescens* have been subjected to.

Naturally, both species express on their membrane Ice-Nucleation Proteins, huge repeats of octapeptides that enhance the nucleation of ice crystals and trigger ice formation very efficiently (Margaritis et al., 1991). The INPs are encoded by the gene InaZ for *P.syringae* and InaW for *P.Fluorescens* (other genes like InaA and InaK have also been identified).

The Ice-Nucleation is believed to be important for these bacteria because of the damages induced by frost to leaves tissues. The wounds permit thereafter the establishment of the bacteria on/into the plant tissues where they can feed on the released nutrients (Margaritis et al., 1991).

It is interesting to think that we consider frost as being a disaster for plants and our species, but by looking at a different perspective, frost can be seen as a very useful weapon for other organisms. What is beneficial or not is just a matter of perspective after all.

The whole idea behind Frostban came from a researcher, Dr. Steven Lindow, from the University of California (Berkeley). It was to knock-out 400 nucleotides of the INPs genes and nullify the ice nucleation activity of the resulting proteins, making the bacteria unable to proceed Ice-Nucleation (Supkoff 1987).

The obtained organisms were called (INA-) as opposed to the wild-type (INA+), INA meaning Ice Nucleation Activity (Skirvin et al., 2000).

After the modification, the resulting organisms were supposed to be sprayed on crops in consequent quantity - 10^3 to 10^8 colony forming units (cfu)/ml (Supkoff 1987) - to outcompete the already present wild-type *P.syringae/P.fluorescens* that had the Ice-Nucleation activity.

Important detail: naturally occurring (INA-) strains of *P.syringae* and *P.fluorescens* do exist. Dr. Steven Lindow did several experiments on pear orchards with *P.syringae* in 1983, without any backslash (Lindow 1987).

The modification was judged necessary because, according to Dr. Steven Lindow (who kindly answered our questions), using a modified (INA+) strain would be better for competition than selecting a naturally occurring (INA-), as they will cover the same ecological niche as the naturally occurring (INA+) bacteria.

This strategy of direct competition is very common in biocontrol. The INRA (Institut National de Recherche Agronomique) and ResaQ Vitibio, the latter managed by Nicolas Aveline (with whom we had the pleasure to have an interview), uses *Aureobasidium pullulans* to outcompete *Botryotinia fuckeliana*, a grapevine pathogen.

Now that we briefly described what was the biological basis of Frostban, we are going to study how AGS managed to test the product, and the results they had from such trial.
Dr. Steven Lindow tested (INA-) bacteria on his side as well.

II. Field tests and results from Dr. Steven Lindow and AGS applications

After many battles and delay of tests (and proof of the non-toxicity of the organisms at first), both Dr. Steven Lindow and AGS obtained the permission to test (INA-) modified bacteria respectively on open fields of potatoes (1985, Tululake) and strawberries (1987, Contra Costa County) in California.

The permissions were given by the National Institute of Health (NIH) and the Environmental Protection Agency (EPA), as well as a Superior Court Judge (Skirvin et al., 2000).

Delays have notably been attributed to violation of EPA rules by AGS in a greenhouse-approved test in 1984 and withdrawal of support of the University of California originally in favor of Dr. Steven Lindow, the latter having to wait for the situation to calm down (Skirvin et al., 2000).

Both tests were carried out in spring 1987 (Skirvin et al., 2000). AGS carried out other tests in winter 1987 and spring 1988.

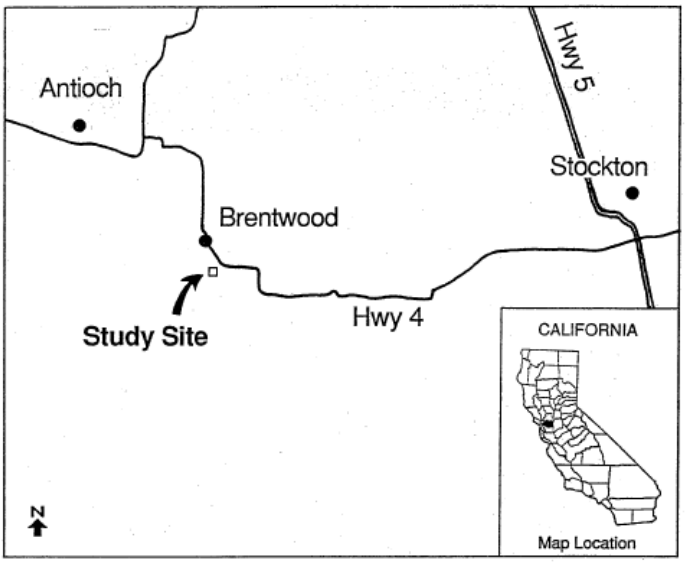
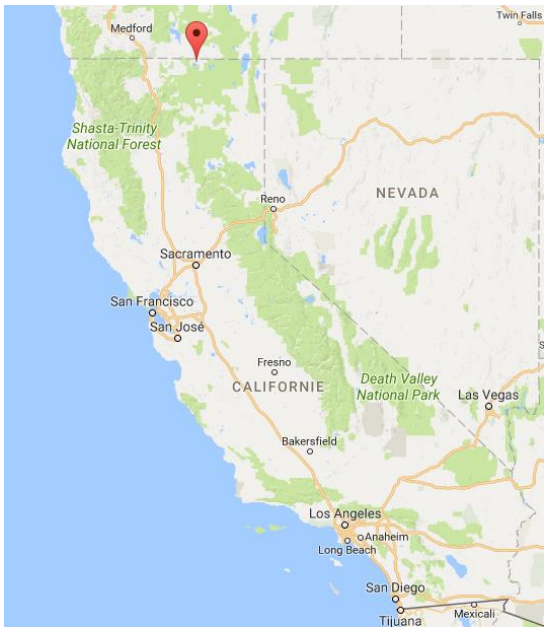


Figure 1. Location of the Frostban® test site in Contra Costa County, California



Contra Costa County and Tulelake, locations of respectively the second AGS Frostban test (winter 1987) and Dr.Steven Lindow's experiments (spring 1987)

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FEB 10 1986

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: 55269-EUP-R/55269-EUP-E (RCB Nos. 416 and 417) — Pseudomonas syringae Strains Cit7dellb (UCB PP1.1) and TLP2dell (UCB PP2.1) on Potatoes - Evaluation of Analytical Methodology and Residue Data (Accession No. 260725)

FROM: Michael P. Firestone, Ph.D., Chemist
Tolerance Petition Section II
Residue Chemistry Branch
Hazard Evaluation Division (TS-769C)

TO: Henry M. Jacoby, Product Manager 21
Fungicide-Herbicide Branch
Registration Division (TS-767C)

and

Toxicology Branch
Hazard Evaluation Division (TS-769C)

and

Toxicology Branch
Hazard Evaluation Division (TS-769C)

and

Frederick Betz
Science Integration Staff
Hazard Evaluation Division (TS-769C)

THRU: Charles L. Trichilo, Ph.D., Chief
Residue Chemistry Branch
Hazard Evaluation Division (TS-769C)

Dr. Steven E. Lindow, Associate Professor of Plant Pathology at the University of California, Berkeley, requests that a waiver for the establishment of a tolerance be granted as per 40 CFR 172.4(b)(2)(iii) for any potato tubers and foliage

Recommendations/Conclusions

Since the proposed use includes a crop destruct clause, RCB considers this a nonfood use and has no objections to granting an EUP.

The authorisation given to Dr. Steven Lindow by the EPA and the Release Control Branch (RCB), permitting him to start his test in 1987, at around the same time as AGS. Adapted from EPA Archive Documents, 1986. EUP: Experiment Use Permit.

We will come back on the context of these field tests later. As for now, let's get to the results.

Firstly, the Dr. Steven Lindow 1987 test, of which the final progress report has been published by the EPA in 1988 (March 22nd). The test, as a reminder, was carried out on a field of potato in Tulelake in 1987.

Unlike Frostban, Dr. Steven Lindow used only *P.syringae* in his trial, of the strains Cit7del1b and TLP2del1. The test was carried out in a field surrounded by a buffer zone. Untreated plants were on the other edge of the buffer zone. Here are the important results:

-After the application, the potato leaves harbored from 10^4 to 10^7 (INA-) bacteria/g of leaf fresh weight. The application happened on April 1987, and in June 1987, only a few of the sprayed bacteria was detectable on the leaves (less than 10/g of leaf fresh weight).

-Only a few (INA-) bacteria (less than 10/g of leaf fresh weight) was detected on the plants that were untreated. Same goes for plants that were located 20-100m to the sprayed zone, as well as insects and harvested potato tubers.

Note than for either the untreated plants or the treated ones, the detection of (INA-) bacteria was more likely due to the naturally occurring (INA-) strains we mentioned earlier. At this time, it was difficult to assess whether or not it was the case.

-During a -3°C frost event, 39-43% of the untreated plants have been recorded to suffer from frost injuries, whereas only 17-21% of the treated plants had suffered due to frost. Another frost event (-5°C) led to positive results as well, with two times more (INA-) treated leaves remaining undamaged as compared to untreated ones, and 4 times more (INA-) treated leaves remaining undamaged as compared to (INA+) treated leaves (this was a positive control).

-Potato tuber yield remained the same no matter the treatment.

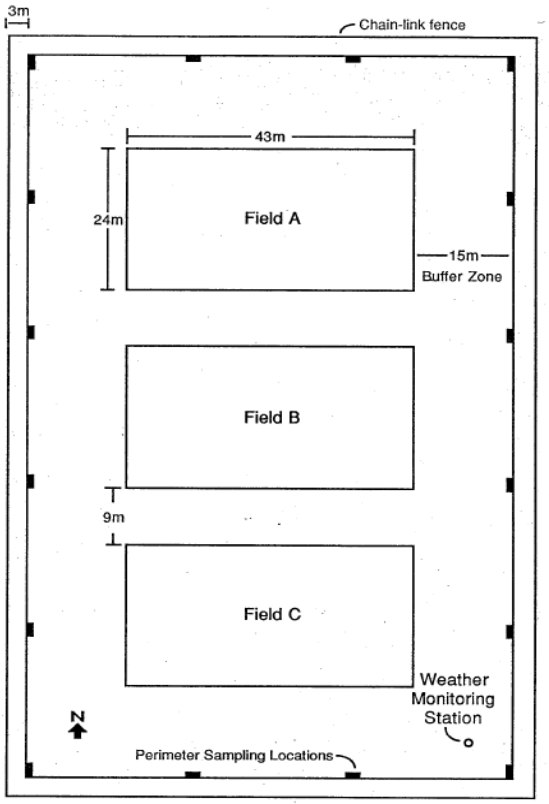
So? Well it seems to have worked and validate the strategy. The treated leaves were less exposed to frost injuries due to the out-competition of the naturally occurring (INA+) *P.syringae.* More encouraging, few or no modified bacteria was found around the treated site. The organisms seem to decline, possibly indicating that the (INA-) phenotype should not be very advantageous for its host.

However, the bacteria could have simply relocated far away, go deep into the soil or try to colonise other environments. The results were in any case encouraging, even though the tuber yield remained unchanged.

However, if the goal was to protect the potato field to favorise a better tuber yield after frost event, which is probably the case, then the treatment can be seen as ineffective.

What about AGS? Here is drawn the line between the public and private sector.
The EPA has indeed published the report of the field study that happened in Contra Costa County in winter 1987, but the results published were only based on the dissemination of the organisms in the surrounding environment (Supkoff et al., 1987).

The actual results of the 1987 and 1988 tests have been relayed only by media (or we just couldn't find them out), possibly because AGS wanted to protect their data, which is totally understandable.
The winter 1987 test was carried out on 3 fields containing 40 plots of 144 strawberries plants.



Strawberry field layout for the experimental application of Frostban®. Fields A,B, and C contained 40 plots, each consisting of approximately 144 strawberry plants.

From Supkoff et al., 1987

Note the weather monitoring station, that measured humidity, rainfall and temperatures (Supkoff et al., 1997), three parameters that we described as crucial for Softer Shock in both "Foliar application" and "Working with the plant" reports. The fence was supposedly used to avoid any intrusion from opposition.

The strains used were the one described at the beginning of the report, *Pseudomonas syringae (strain RGP 36R2)*, and *Pseudomonas fluorescens (strain GJP 17BR2)* (Supkoff et al., 1987).

The product was sprayed using backpack sprayers (Supkoff et al., 1987). The cells were diluted in water at a concentration of 10^8/ml, giving an average of 10^7 colony forming units per leaves (Supkoff et al., 1987).

Different combinations of strains, phenotypes (for control, like Dr.Steven Lindow did), and untreated plants were made according to each fields A/B/C (Supkoff et al., 1987).

The conclusion of the EPA of the field study was, after samplings of air, plants and soil in the test environment and its surrounding:

> While genetically engineered bacteria were recovered from air and off-site vegetation samples, these detections were limited to a relatively low percentage of total samples. The pattern of detection of Frostban® bacteria in samples of air and vegetation suggests that off-site movement of these bacteria was likely due to aerosol drift during microbial pesticide application.

From Supkoff et al., 1987

30 pages of analysis and one conclusion, organisms were found out of the target field, and spray drifting was the main responsible.

The EPA didn't seem to give any negative recommendation as for the banishment of Frostban and its non-commercialisation.

This is all summed up of course, we encouraged reader to look upon the full report of (Supkoff et al, 1987) to have all the results.

As for the results communicated by AGS after their three tests:

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Genetic Engineering

Advanced Genetic Sciences Inc

Bacteria

Altered Bacterium Does Its Job : Frost Failed to Damage Sprayed Test Crop, Company Says

June 09, 1987 | THOMAS H. MAUGH II | Times Science Writer

Advanced Genetic Sciences Inc. announced Monday that its Frostban bacteria have successfully protected strawberries from below-freezing temperatures and that the genetically altered bacteria did not escape from the test plot during the six-week trial.

The New York Times

Business Day

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ALTERED BACTERIA FIGHT FROST

AP
Published: June 10, 1987

OAKLAND, Calif., June 8— Vandalized strawberry plants and hot weather disrupted the first outdoor test of frost-fighting bacteria, but the company that invented it said preliminary results show success.

Positive results communicated by AGS after the spring 1987 test. From [Los Angeles times](#) and [New York times](#)

{BLR 766} Environmental Release - Ice Nucleation - Advanced Genetics Sciences.

BLR UPDATE: ADVANCED GENETIC SCIENCES ANNOUNCES COMPLETION OF SECOND FIELD TEST
—Genetically Altered Bacteria Successfully Combat Frost

OAKLAND, CA 4/27/88—Advanced Genetic Sciences (AGS) announced this date the successful completion of its second field test of its genetically altered bacteria (Frostban), designed to prevent frost damage to fruit and nut crops. The bacteria, which has one gene deleted, do not secrete proteins that contribute to the formation of frost, unlike the strains that usually colonize on plants.

Preliminary results confirmed that Frostban effectively protected strawberry plants from the natural bacteria that cause frost to form. According to Joseph Bouckaert, president and chief executive officer of AGS, the company has concluded that at least one of the strains tested will be further developed for product registration.

The first experiment, begun in April and completed in June 1987, demonstrated that Frostban successfully survived on the plants. Thus, it became the world's first outdoor field test of a genetically altered bacteria. See <BLR 650>(May-June 1987).

The second experiment was designed to test the ability of two strains of Frostban to protect strawberry blossoms against frost and to monitor the growth and of the bacteria. The tests also allowed scientists to determine the most effective dosage, frequency and timing for applications of Frostban.

Published in the BLR, 1988, this confirms that AGS announced positive results for both of their 1987 tests.

Overall this shows that, according to Dr.Steven Lindow's experiments and what claimed AGS, Frostban was a promising product, that had surely its flaws as the EPA proved that drifting could induce organism unwished releases, but great qualities as well.

But many things went wrong with Frostban, AGS, Dr. Steven Lindow, and the locals. We are going now to assess how society received such project of GMO use and draw comparison between Softer Shock and Frostban, to give an insight on the evolution of GMOs and synthetic biology in 30 years.

III. Societal tensions raised by Frostban and (INA+) modified bacteria

More than just a scientific result and a proof of concept, the field tests provided insight also on whether or not the society was ready for such product and how much of a cleavage there was between AGS, Dr. Steven Lindow and fierce opposition led by Mr.Jeremy Rifkin, an American social theorist and economist followed by locals.

The opposition first started legally with the leading of Mr.Jeremy Rifkin in April 1984, willing to prevent Dr.Steven Lindow experiments on potatoes. His arguments were about the unsatisfying risks assessment made by the NIH and that a modified *P.Syringae*, as it is normally a bacteria implied in the water cycle and formation of clouds, could perturbate rainfalls patterns (Skirvin et al., 2000). After many debates, Dr.Steven Lindow's test was postponed.

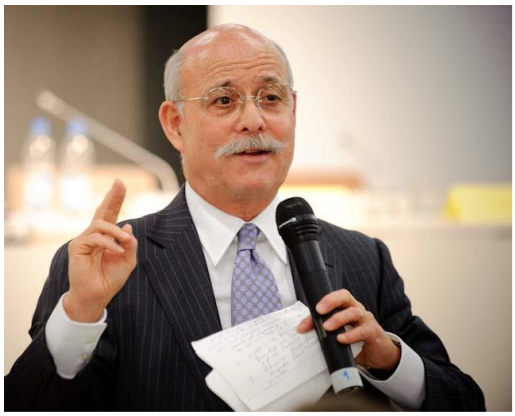
Following was the AGS request for its first test of Frostban (originally planned in 1984 in Monterey County). The request was approved by the EPA but residents wanted to forbid the test because, as said earlier in this report, AGS already did a Frostban test in greenhouses, and violated the containment rules set by the EPA.

Even if EPA later accepted the proofs that no GMO had been released out of the greenhouses, the event of rule violation gave such bad publicity to AGS that their new request for open field test backfired (Skirvin et al., 2000). Seeing that locals at Monterey County clearly showed signs or fierce opposition, and after cumulating bans and delay of their test there, AGS turned to Contra Costa County.

There, they managed to set a test on 2400 strawberries plants on April 1985 but an environmentalist group, the Berkeley Greens, got through the fences and uprooted 2200 of the 2400 strawberries plants in the field (Skirvin et al., 2000).

The same year in May, Dr.Steven Lindow obtained his permission for his tests, but a petition from locals and bad publicity from recent events led to the withdrawal of the University of California (one of his support) and the delay of the test (Skirvin et al., 2000).

Two years later, Dr.Steven Lindow's test was finally approved by the University, and AGS finally won legally its battle against Mr.Jeremy Rifkins in April 1987 (Skirvin et al., 2000).
Both Dr.Steven Lindow and AGS had finally what they wanted, 3 years and many harvested tensions later.



Mr.Jeremy Rifkin, the EPA, and the University of California (Berkeley), three entities that have influenced the course of events for (INA-) bacteria field testing.

The author of the report you are currently reading was not born at this time, so I decided to contact directly Dr. Steven Lindow and ask him how he felt about the whole situation. He very generously answered:

"In general, it was my impression that most members of the general public are interested in and enthusiastic about advances in agricultural biotechnology such as our production of ice nucleation deficient strains of bacteria, but that there was very vocal opposition from a small subset of society which carried a lot of weight and got a lot of attention. I had thought that with more examples of the benefits of biotechnology and of education on how biotechnology is involved in our society, it would generally become accepted. As a group, I think the answer is generally yes that they are accepted, but there still remains very vocal opposition, by a small group of people who want to keep this issue and the public side. This has led to continued strict regulation as well as a lot of press coverage of the opposition. "

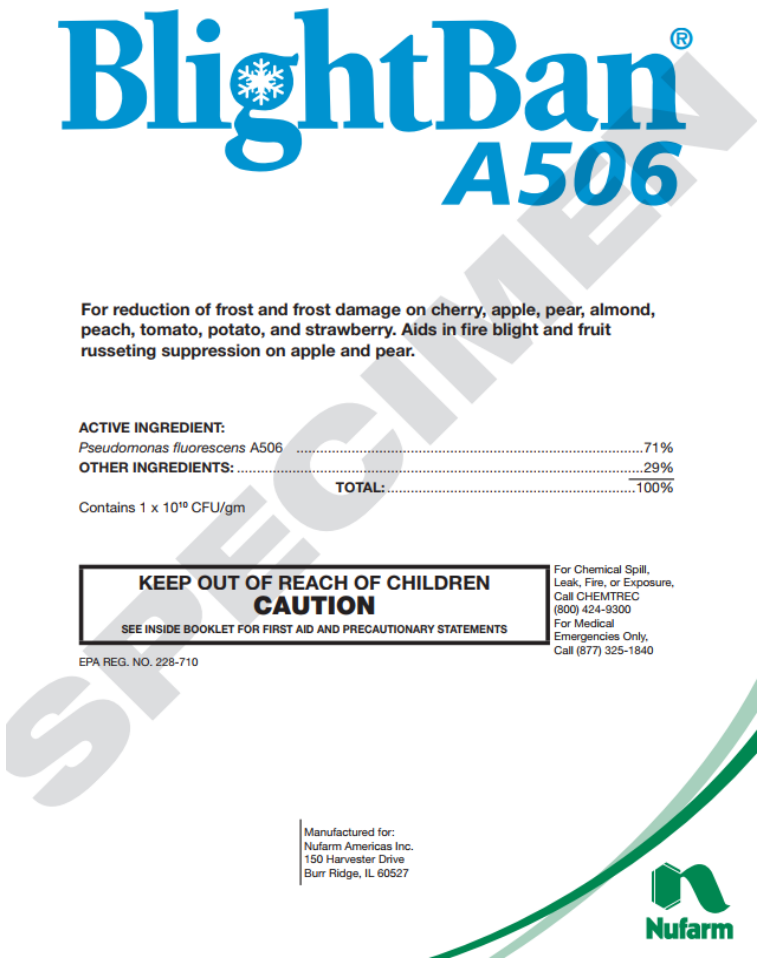
-Mr Steven Lindow, 2017

More education from AGS and less press coverage for opposition could have led to the final development of Frostban as it was at this time.

Important enough is the fact that the product was kept by AGS (who merged with DNAP in 1987) and four new different formulations were registered, using.... wild-type (INA-) bacteria, much easier to register as a product than with a GMO product.

The Frostban development was then sold to Frost Technology Corporation in 1992, and the license was dropped after one year.

Then Plant Health Technologies picked the license, decided to diverge from exclusively treating frost injury and treat fire blight with the *P.fluorescens A506* that was used in the Frostban formulations containing wild-type (INA-) bacteria. The name was changed to Blightban A506 and was sold as a treatment for fire blight on crops, as well as frost damages reducer (Skirvin et al., 2000). The product still exists and is commercialized by Nufarm, ironically for treatments of strawberries...and potatoes. Organisms are not the only entities that mutate…


BlightBan®
A506

For reduction of frost and frost damage on cherry, apple, pear, almond, peach, tomato, potato, and strawberry. Aids in fire blight and fruit russeting suppression on apple and pear.

ACTIVE INGREDIENT:
Pseudomonas fluorescens A506 ....71%
OTHER INGREDIENTS: 29%
TOTAL: 100%

Contains 1 x 10^{10} CFU/gm

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CAUTION
SEE INSIDE BOOKLET FOR FIRST AID AND PRECAUTIONARY STATEMENTS

For Chemical Spill, Leak, Fire, or Exposure, Call CHEMTREC (800) 424-9300
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EPA REG. NO. 228-710

Manufactured for:
Nufarm Americas Inc.
150 Harvester Drive
Burr Ridge, IL 60527

Nufarm

Blightban A506 by Nufarm, the current aspect of Frostban 30 years later. The snowflake symbolizes a distant echo from the past.

IV. Comparison with Softer Shock Applied Design

To understand properly this part, we recommend reading our applied design reports where we try to give final composition of our product and how it is going to be applied, as well as risks managements and assessments.

The goal here is to show how insights on development of GMO crop spray have evolved in 30 years. In no way, we pretend Softer Shock is better than Frostban. We can't assess such things because the context of both projects is sensibly different, and our applied design was made based on our experience, which is certainly lower than the one of the AGS team that developed Frostban.

However, here is a table summarizing the differences between both products, remember we are talking about Frostban and not Blightban A506:

	Frostban	Softer Shock
Chassis selection	*P.syringae* and *P.fluorescens*, two bacteria of the plant microbiota found in various environements	Chassis selection proceeded by metagenomics for personalised treatment or use of common biocontrol strains
Killswtich and safety	No killswitch, buffer zone during tests, weather monitoring	Synthetic auxotrophy, direct killswitch, gene transfer avoidance mechanism. Physical containment with tunnel sprayer and adjuvants
Toxicology study	Toxicology study made by EPA and NIH and validation of non-pathogenicity for humans	Theoretical toxicologic and ecotoxicologic study but no real tests for now
Spraying technique	Use of backpack sprayer	Tunnel sprayer
Stress treated	Frost prevention	Frost prevention and heat protection
Medium	Water	Adjuvants, encapsulated organisms and synthetic amino acid
Biological mechanism	Competition with (INA-) phenotype.	Anti-Freeze Proteins/Ice-Nucleation Proteins for frost and Chitin/Casein for sunlight
Method of modification	Genome modification (Knock-out)	Use of plasmid vector and genome modicfication
Field study and empirical tests	Three field studies from AGS (Mr.Steven Lindow did not use Frostban)	None

The major flaw of our product we can observe in this comparison is of course the absence of field study on both toxicology and efficiency of the product, which is a very important part.

Everything about Softer Shock sounds great as compared to Frostban, but it is all theoretical, and we do not for now have a reliable proof of concept, neither have predicted precisely the cost of industrial production and conservation of our product.

We want a lot of things for Softer Shock, and some of them will more likely not be possible, but with this table we can show how much things have evolved.
With the context of the IGEM and modern society, 30 years after the Frostban first field application, questions like biosafety, integration of microbiota, and techniques of modifications have changed.
Just the biosafety itself shows how the scientific community tries to correct the past flaws and integrate GMOs and synthetic biology in society.

Note that differences between the bioreactor we predicted to use in our entrepreneurship parts and Frostban are even more pronounced, and comparison of both is kind or irrelevant, since they do not belong to the same category.

We will finish this report by quoting Dr.Steven Lindow, with the answer he provided us about how synthetic biology and GMOs could be accepted nowadays (as Softer Shock) :

“Most people are not willing to accept any risk if they do not see some particular benefit and many environmental and agricultural applications have no immediate benefit to the lay public, and so therefore they are rather reluctant to accept even minimal possibilities of risk.”

-Dr.Steven Lindow, 2017

We want to thank Dr. Steven Lindow again for his answers to our questions.

Thank you.

The IGEM IONIS Team

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