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(54) Title: IMPROVEMENTS IN OR RELATING TO GENE SILENCING

(57) Abstract: The present invention provides a pesticidal composition comprising a double-stranded ribonucleic acid (dsRNA) and a carbohydrate, wherein said dsRNA is capable of RNAi gene silencing and comprises a strand that is complementary to at least part of a nucleotide sequence of a gene from a plant pest organism.

IMPROVEMENTS IN OR RELATING TO GENE SILENCING

The present invention relates to the control of gene expression using double stranded RNA (dsRNA). In particular, the invention relates to compositions which enhance the uptake of dsRNA administered external to a target organism to enable effective gene silencing in that target organism.

RNA interference (RNAi) is a well-established technique used to down regulate gene expression by using dsRNA or small interfering RNA (siRNA) to trigger degradation of mRNA of a particular gene of interest, thus preventing translation. RNAi has not only provided a means of functionally analysing genes, but has been used for the control of pest organisms. A problem with the application of dsRNA for the control of pest organisms concerns the poor uptake of dsRNA by a pest organism when the dsRNA is applied exogenously at a locus at which they exist.

Exogenous dsRNA refers to dsRNA which is applied to a target organism such that it is incorporated into a target organism and results in the effective silencing of an essential target gene. The skilled artisan will be aware that dsRNA may be exogenously applied in a composition in its native form, or applied whilst incorporated in an organism different to that of the target organism.

There has been some limited success in targeting organisms with dsRNA by topical application of compositions comprising dsRNA onto a food source of the target organism, wherein the dsRNA is ingested by the organism, resulting in effective silencing of an essential gene target.

Whilst this technique has provided an efficient means of dsRNA control in a number of target organisms, such as coleopteran pest species, many other pest species are refractory to this method of dsRNA delivery. Therefore the application of dsRNA for use as a pest control agent is significantly limited.

The present invention provides at least to some extent a solution to the problem of exogenous dsRNA delivery in those pest species which are refractory to those methods presently used in the art.

According to the present invention there is provided a pesticidal composition comprising a dsRNA and a carbohydrate, wherein said dsRNA is capable of RNAi gene silencing and

comprises a strand that is complementary to at least part of a nucleotide sequence of a gene from a plant pest organism.

In a preferred embodiment of the invention the composition of the invention is substantially devoid of salts.

- 5 The composition may comprise additional components, suitable components having a function of a binding agent, and/or an anti-desiccant and/or a humectant a rheology modifier and/or a bulking agent.

Suitable binding agents include emulsifiable mineral oil (Actipron), carrageenan, molasses, starch, dextrine, ethyl cellulose, methacrylate polymers, gelatine, glucose, arabic gum or other
10 vegetable gums, tragacanth gum (dried sap), honey, whole or skimmed milk, polyvinylpyrrolidone, acrylic polymers, carboxymethyl cellulose and Lysine.

Suitable anti-desiccants include calcium alginate, water absorbing polymers, cellulose, glycerol and molasses

Suitable humectants include glycerol, polyacrylamide gel, adipic acid, alginic acid, acetamide-B, mercaptoethylamide, boric acid, calcium stearate, cellulose acetate, stearic acid or calcium
15 stearate, gellan, alginate, polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone and polyvinylpyrrolidone/vinyl acetate copolymer.

Suitable rheology modifiers include water absorbing polyacrylamide (Alcosrob AB3), calcium, alginate, dextran, pre gelatinised corn flour, carob gum, sodium carboxymethylcellulose, fumed
20 silicon dioxide calcium gluconate, acacia gum or xanthan gum.

Suitable bulking agents include attapulgit clay, bentonite clay, kaolinite, calcium montmorillonite, calcium carbonate, celatom, celite, ground clay, kaoline, diatomaceous earth and ground limestone.

The skilled artisan will recognize components may have more than one function.

- 25 The following tables provide examples of suitable formulations of the present invention.

Function	Example compound	Amount (%)
Binder	Starch	2-3
Adjuvant	Sucrose	10-15
Carrier/Ai	Bacterial biomass containing active (dsRNA)	83-74
	Humidity of product	5-8

Function	Example compound	Amount (%)
Anti-desiccant	Sodium alginate	1-5
Adjuvant	Sucrose	10-15
Carrier/Ai	Bacterial biomass containing active (dsRNA)	84-72
Humectant	Sorbitol	1-2
	Humidity of product	5-8
Function	Example compound	Amount (%)
Rheology modifier	Sodium alginate	0.5-2
Adjuvant	Sucrose	10-15
Carrier/Ai	Bacterial biomass containing active (dsRNA)	30-40
Bulking agent	Micronized cellulose	54.5-65
	Humidity of product	5-8

In an aspect of the invention the pesticidal composition of the invention comprises a dsRNA which is synthetically transcribed in vitro.

- 5 In another aspect of the invention, the pesticidal composition of the invention comprises a dsRNA which is transcribed by a microorganism. Preferably, when present in the composition, the microorganism is inactivated.

By 'inactivated' it is meant that the microorganism is treated by heat or with a chemical such that it is no longer capable of propagation.

Suitable microorganisms include those selected from the group of bacteria, fungi, algae and yeast.

Often inactivation of a microorganism will result in cell lysis. Accordingly, in an aspect of the invention the dsRNA is present in the composition as a component of a cell lysate. The cell
5 lysate may be a complete lysate, characterized in that all cells have undergone lysis, or the lysate may contain a proportion of intact cells. Therefore, dsRNA of the invention may be present in a cell lysate, intact cells or a proportion present in both.

Preferably, the carbohydrate present in the pesticidal composition is provided at a concentration that is sufficient to promote the plant pest organism to take up dsRNA to enable effective RNAi
10 gene silencing. In particular it is preferred that the carbohydrate is present in the composition at an amount from 0.01 % to 20% of total volume of the composition.

In another aspect of the invention the carbohydrate comprises at least one glucose residue and/or at least one fructose residue. In particular, the carbohydrate is sucrose, maltose, glucose, fructose or raffinose.

15 In another aspect of the invention the carbohydrate is a sugar alcohol. Suitable sugar alcohols include sorbitol, mannitol and myo-inositol.

In a preferred aspect of the invention the plant pest organism is of the order Hemiptera.

The Hemiptera plant pest may be selected from the group consisting of green stink bug
Acrosternum hilare (Say), Brochymena quadripustulata (Fabricius) rough stink bug, Chlorochroa
20 sayi (Stål) Say stink bug, Coptosoma xanthogramma (White) black stink bug, Euschistus servus (Say) brown stink bug, Euschistus tristigmus (Say) dusky stink bug, Euschistus variolarius (Palisot de Beauvois) onespotted stink bug, Halyomorpha halys (Stål) brown marmorated stink bug, Nezara viridula (Linnaeus) southern green stink bug, Oebalus pugnax (Fabricius) rice stink bug, Perillus bioculatus (Fabricius) twospotted stink bug, Piezodorus guildinii (Westwood)
25 redbanded stink bug, Plautia stali Scott oriental stink bug, Thyanta custator accerra McAteered shouldered stink bug, Euschistus heros Neotropical brown stink bug, Dichelops melacanthus.

In another aspect of the invention it is envisaged that the pesticidal composition of the invention comprises at least one other pesticidally active ingredient in combination with the dsRNA of the invention. The pesticidally active ingredient may target the same plant pest organism or a

different plant pest organism. Suitable pesticides are those commonly known to the skilled artisan.

The present invention further provides a method for controlling a plant pest organism comprising applying to the plant, to the seed of a plant, or locus of a plant or seed the composition of the invention. Preferably the composition is ingested by a plant pest organism.

Another aspect of the invention relates to a method for controlling a plant pest organism comprising applying to a plant, sequentially and in any order, a dsRNA capable of RNAi gene silencing comprising a strand that is complementary to at least part of a nucleotide sequence of a gene from a plant pest organism, a carbohydrate and optionally another pesticidally active ingredient.

The present invention also relates to the use of the composition of the invention for controlling plant pest growth and/or infestation.

The present invention also relates to the use of a carbohydrate, in a composition comprising pesticidal dsRNA, to satiate the appetite of a pest so that the production of salivary nucleases by the pest is substantially inhibited. In particular, the carbohydrate is sucrose, the pest is a plant pest, the plant is a soybean plant, the pest is a stink bug and the carbohydrate is present in the composition at an amount of 0.01% to 20% of the total volume of the composition.

The present invention also relates to a method of substantially inhibiting the production of salivary nucleases in a pest fed a composition comprising a pesticidal dsRNA by including a carbohydrate in the composition. In particular, the carbohydrate is sucrose, the pest is a plant pest, the plant is a soybean plant, the pest is a stink bug and the carbohydrate is present in the composition at an amount of 0.01% to 20% of the total volume of the composition.

Examples

The invention is further exemplified by the following non-limiting examples.

Materials and methods

Insect rearing and staging of Stink Bugs.

The precise conditions involved in producing a Stink Bug “test” insect depends on the specific Stink Bug. For example Stink Bugs were reared at 26°C, 65% relative humidity, with 16:8 hours light: dark cycle. Adults were kept in insect cages (50 cm x 50 cm x 80 cm) and fed with fresh

runner beans -*Phaseolus coccineus*- and crushed peanuts- *Arachis hypogaea* L. A soybean plant- *Glycine max*- served as an extra food source and oviposition substrate. Every 2 weeks, fresh eggs were collected from 3 cages. Egg masses were cut out of the plant leaves, and used to seed a container (15 cm x 25 cm x 11 cm) lined with paper tissue, closed with a mesh and stored in the rearing chamber (26°C, 65% RH, with 16:8 hours l: d). Runner beans - *Phaseolus coccineus* - were added when first instars appeared. From the third instar onwards, a tray containing a cotton boll soaked in tap water and a tray containing crushed peanuts was added. When the nymphs molted to fourth instar, they were transferred to larger boxes (27 cm x 27 cm x 13 cm), with double food/liquid supply than the younger stages. Every 3 days, tissue, beans, peanuts and water were refreshed. When nymphs developed into adults, 300 adults were put in a cage (3/2 males/females) and maintained as previously described. For bio-assays, eggs less than 24h old were collected from soy bean plants from 3 cages and maintained in boxes, as previously described, until reaching second instar. These second instar nymphs were subsequently used in bio-assays described below.

Alternatively Stink Bugs were reared at 26°C, 65% relative humidity, with 16:8 hours light: dark cycle. Adults were kept in insect cages (30cm x 30cm x 30cm) and fed with fresh runner beans - *Phaseolus coccineus*-, sunflower seeds- *Helianthus annulus* - and a tray containing a cotton boll soaked in tap water. Eggs were laid on the mesh and sleeve of the cage. Every 3 days, eggs were removed from 3-5 cages, seeded in a petridish (Greiner) on a filter paper (Whatman), closed with breathable tape (URGO) and stored back in the rearing chamber (26°C, 65% RH, with 16:8 hours l: d). Runner beans - *Phaseolus coccineus* - were added when first instars appeared. Second instars were transferred to boxes containing runner beans on a paper tissue and boxes were closed with a mesh. From the third instar onwards, a tray containing a cotton boll soaked in water and a tray containing sunflower seeds was added until adulthood. Every 3 days, tissue, beans, seeds and water were refreshed.

When nymphs developed into adults, 100 adults were put in a cage (3/2 males/females) and maintained as previously described. For bio-assays, eggs were staged by color (eggs turn orange the day before hatching) and maintained in boxes- as previously described- until reaching second instar.

Carbohydrate in vitro assays

General description in vitro assay

Stink Bug nymph in vitro assays were performed in 96-well nylon mesh filter plates (Millipore). Each well of a 96-well filter plate houses a second instar nymph exposed to a parafilm sachet containing the food source. The filter at the bottom of the 96-well plate ensured aeration of the well. Feeding of nymphs occurred by piercing the stylet through parafilm sachets and sucking
 5 up the fluid. Feeding sachets were made by placing a parafilm strip (15.5cm/11cm) on a 96-well filter plate (Millipore) which is placed on a vacuum manifold (Millipore) and connected to vacuum pump (Laboport). The vacuum pump was switched on and when wells were slightly depressed, extra pressure was given by rolling a 96-well rubber mat on top of the parafilm to form 96 U-shaped wells. When the vacuum manifold was switched off, wells were filled with 25 µl of
 10 feeding solution (carbohydrate + dsRNA), sealed with an Ampliseal (Greiner bio-one) and removed from the manifold. One second instar nymph was placed in each well of a 96-well plate (Millipore) and the plate was closed with the feeding sachet, 1 -precut- blotting paper of 8/12 cm (Bio-Rad) and the lid of the 96-well plate. In an experiment 24 nymphs were tested per condition. Plates were put in plastic boxes on a metal mesh and stored in the rearing chamber
 15 (26°C, 65% RH, with 16:8 hours l: d). Feeding solution was replaced with fresh rearing (complex) diet (BioServe) in the absence of dsRNA, 3 and 6 days post exposure to dsRNA.

Complex diet was prepared according to manufacturer's description and used within 7 days of preparation. The nymphal survival rate was recorded from 3 to 10 days post exposure to dsRNA. A lethal RNAi effect was expected from day 6 onward. The assay system was
 20 validated using dsRNA corresponding to a known target as the positive control and a fluorescent protein dsRNA (GFP) as the negative control. The positive control for testing the different carbohydrates was dsRNA made up in 15% carbohydrate (in particular sucrose) solution, and the negative control for the carbohydrate testing was dsRNA made up in ultrapure water (MilliQ).

25 Carbohydrate in vitro assays with synthetic dsRNA

dsRNA production

To generate the dsRNA template, material was generated by PCR using two target specific primers containing the T7 RNA polymerase promoter sequence at their 5'-end. The PCR reaction was analyzed on agarose gel and purified using the QIAquick PCR purification kit
 30 (Cat.n°28006, Qiagen). The purified template was quantified using the DropSense96 (Trinean). The template was used in an in vitro transcription reaction (RiboMAX™ Large Scale RNA Production System–T7 P1300, Promega). To generate at least 1mg purified dsRNA an

equivalent of 20 reactions was set-up and incubated overnight at 37°C. The single-stranded RNAs produced by transcription from the templates were allowed to anneal, were then treated with DNase and RNase, and were purified by precipitation. An extra purification step using a VIVAPSIN20 column with MWCO 30kDA (VS2022, Sartorius) was included for the removal of residual salt and unincorporated nucleotides. As a final quality check the samples were analyzed on agarose gel.

Bioassay 1

In a first round of testing, 2 monosaccharides, 2 disaccharides, 1 oligosaccharide and 3 carbohydrate alcohols were tested for their potential to elicit an RNAi response in second instar nymphs upon feeding. All carbohydrates were tested at a final concentration of 15% (m/v %) in combination with synthetic dsRNA at a final concentration of 1 µg/µl.

Monosaccharides tested were glucose and fructose. Disaccharides tested were sucrose (control), and maltose. The tested oligosaccharide was raffinose and carbohydrate alcohols were mannitol, myo-inositol and sorbitol. Two feeding mixtures per carbohydrate were prepared by mixing 390 µl 25% carbohydrate solution with 260 µl 2.5 µg/µl solution of dsRNA. All solutions were prepared in Ultrapure water (MilliQ). The control solution contained GFP synthetic dsRNA and the target solution contained positive control synthetic dsRNA, which were both diluted in MilliQ. Feeding assays were set up and nymphal survival was recorded as described above.

As shown in Fig.1, no RNAi effect was observed when nymphs were exposed to synthetic dsRNA in the absence of a carbohydrate.

Bioassay 2

In a second round of testing, all carbohydrates (except myo-inositol) used in the first bioassay, were tested at a concentration of 15%, 10%, 5% and 1% (m/v %) in combination with synthetic dsRNA at a final concentration of 1 µg/µl.

Starting from a stock solution of 25% for each carbohydrate, dilutions of 16.6%, 8.3%, 1.6% were made. Two dsRNA solutions at a concentration of 2.5 µg/µl were prepared: the control solution containing GFP synthetic dsRNA and the target solution containing positive control synthetic dsRNA. All dilutions were made up in ultrapure water (MilliQ).

The dsRNA solutions and carbohydrate stocks were combined- as described in the first round of testing i.e. to a final concentration of synthetic dsRNA of 1 µg/µl and carbohydrate

concentrations of 15%, 10%, 5% and 1%. Feeding assays were set up with second instar nymphs and nymphal survival was recorded as described above.

As shown in Fig.2 to 5, an RNAi response was observed when nymphs were exposed to synthetic dsRNA in the presence of sucrose, glucose, fructose and maltose at all concentrations tested. When dsRNA was provided in raffinose, the RNAi effect could only be achieved when using a final concentration of 10% and 5 % (m/v %) (Fig.6). At the given concentrations, other carbohydrates were toxic (high mortality during the first days of feeding) or their presence was unable to induce a clear RNAi response in the nymphs (Fig.7, Fig.8). An RNAi response may be observed if lower concentrations were used.

10 Carbohydrate in vitro assays with bacterially produced dsRNA

The sequence of interest was cloned as a hairpin (inverted repeats separated by a spacer sequence) behind an IPTG-inducible promoter. The resulting dsRNA expression plasmid was then transformed into a suitable bacterial host. The production of dsRNA was done by fermentation. The bacteria containing the dsRNA expression plasmid were grown in defined medium to a specific optical density and induced by addition of IPTG for a specific time period. After induction, the bacteria were harvested by centrifugation, resuspended in water to a defined optical density and inactivated by heat treatment.

Bioassay

Carbohydrates sucrose, maltose and lactose were tested in combination with bacterial lysate expressing a fluorescent protein dsRNA (GFP) and target dsRNA fragment. Carbohydrates were tested at 15% (m/v %) and 5% (m/v%) final concentrations. Bacterial lysate was tested at concentrations of 0.5U, 0.25, 0.125 and 0.0625U per nymph 20U/ml, 10U/ml, 5U/ml and 2.5U/ml respectively. 1 unit (U) corresponds to the amount of bacteria present in 1 ml culture with an OD600 nm value of 1 prior to (heat) inactivation. The tested carbohydrates were made up in solutions of 30% and 10% (m/v) concentrations. Bacteria expressing GFP and target dsRNA were diluted to solutions of 40U/ml, 20U/ml, 10U/ml and 5U/ml. All dilutions were made in ultrapure water (MilliQ). Carbohydrate solutions and bacterial lysate suspensions were mixed in equal proportions i.e. 1:1. Feeding assays were set up on second instar nymphs and nymphal mortality was recorded as described above.

As shown in Fig.9 to Fig.11, a clear RNAi response is visible when nymphs were exposed to all concentrations of bacterial lysate containing the target dsRNA mixed with both dilutions of

sucrose and maltose (5% and 15%). No RNAi response was seen when nymphs were exposed to the bacterial lysate in combination with 15% and 5% lactose (Fig.13-Fig.14).

Carbohydrate on planta assays with bacterially produced dsRNA

Uptake bioassays

- 5 Soy bean seeds (Jack variety) were grown at 26°C with 16:8 hours light: dark cycle. Seeds were sown in 9-cm potting containing Universal potting soil (AVEVE) and Osmocote (EVERRIS). Pots were lined up in a tray (30cmx28cmx6cm), placed in the growing chamber and watered daily until reaching V1-V2 development stage. Two solutions of Fluorescein isothiocyanate–inulin (FITC inulin, Sigma) were prepared in MilliQ and in 60% sucrose. Both FITC inulin solutions
- 10 were mixed with bacterial lysate expressing non targeting GFP, resulting in a solution with a final concentration of 75U/ml bacterial lysate, 2 mg/ml FITC inulin and 15% sucrose and a solution with a final concentration of bacterial lysate of 75U/ml and 2mg/ml FITC inulin in the absence of a carbohydrate.

- In each condition, 1 ml of the solution was sprayed on the leaves (adaxial and abaxial surfaces),
- 15 buds and stem of each plant and replicated 3 times. Sprayed plants were individually placed on the lid of a 9-cm petri dish (Greiner) and a Plexiglas cylinder (diameter 9.2cm) was placed over the plant and petri dish.

Individual experiments were performed using various varieties of Stink Bug nymphs. The nymphs were reared as previously described until reaching second instar.

- 20 Eight second instar nymphs, were placed on each sprayed plant and a nylon mesh was placed over the cylinder to permit aeration, reduce condensation inside and prevent nymphal escape. Plants were placed on a tray and stored at 26°C, 65% relative humidity, with 16:8 hours light: dark cycle.

- Three days post infestation, the 8 nymphs from each plant were pooled in a 1.5 ml vial and
- 25 washed twice with MilliQ. The nymphs were placed on a tissue to dry and individually transferred to a single well, containing a metal bead, of a deep-well plate containing 1 ml tubes-strips (Machery -Nagel). The plates were flash-frozen in liquid nitrogen and placed in the Tissue-Lyzer (Qiagen) for 1 min at 30 Hz. After maceration of the nymphs, the plates were centrifuged for 10 minutes at 4000 g, 200 µl MilliQ was added to each well and mixed.
- 30 were centrifuged a second time for 10 minutes at 4000 g. Twenty µl of each well was transferred

twice to a 348 well plate (Greiner) and fluorescence was measured at 480nm (Ext) 530nm (Em) using the Clariostar microplate reader (BMG Labtech). Each data point represented the average of 2 measurements, 24 datapoints per treatments were obtained.

High uptake of FITC inulin by second instar nymphs was observed when nymphs had been feeding on plants sprayed with a suspension of bacterial lysate, FITC inulin in 15% sucrose. No to very low uptake of FITC inulin by the nymphs was observed when plants were sprayed with a suspension of bacterial lysate and FITC inulin in the absence of sucrose (Fig.15).

Nymphal survival bioassays (continuous exposure)

Jack soy bean seeds were grown until reaching V2-V3 development stage as previously described. The plants were sprayed with a 15% carbohydrate solution eg.sucrose, containing 75U/ml bacterial lysate expressing non target GFP dsRNA and a solution containing 75U/ml bacterial lysate expressing non target GFP dsRNA in the absence of a carbohydrate. For each condition a total volume of 2 ml was sprayed on the plant and replicated 3 times. Thirty second instar nymphs, were placed on each sprayed plant. Spraying of the plants, rearing of insects, caging and storage of the plants was performed as previously described.

The nymphal survival rate was recorded from 3 to 10 days post exposure to the bacterial lysate. Clear differences in survival rate of the nymphs were demonstrated in this experiment. A very low survival rate of nymphs was observed on plants sprayed with bacterial lysate expressing non target GFP dsRNA in the absence of sucrose (Fig.16). In contrast, the nymphs on plants sprayed with the bacterial lysate in the presence of sucrose could survive throughout the assay.

These findings, together with the results of the uptake experiments previously discussed, suggest that presence of sucrose stimulates feeding and is required for a normal development of the nymphs.

As a consequence of this experiment, RNAi effects with bacterial lysate expressing lethal target dsRNA were performed in the presence of a carbohydrate, which was selected as a positive control for subsequent assays.

RNAi on planta (3 days exposure)

Jack soy bean seeds were grown until reaching V2-V3 stage as previously described. The plants were sprayed with a 15 % sucrose solution containing 75U/ml bacterial lysate expressing non targeting GFP or target dsRNA. For each condition a total volume of 2 ml was sprayed on

the plant and replicated 3 times. Thirty second instar nymphs, were placed on each sprayed plant. Spraying of the plants, rearing of insects, caging and storage of the plants was performed as previously described.

- 5 Three days post infestation, the 25 nymphs from each plant were transferred to a 20-cm petridish (Greiner) containing a filter paper (Greiner) and a runner bean (*Phaseolus coccineus*). The nymphal mortality was recorded from 3 to 10 days post exposure to the AI.

As shown in Fig.17 and Fig.18, spraying plants with a 15% sucrose suspension of bacterial lysate expressing target dsRNA led to a dramatic increase in insect mortality when compared to GFP control.

10 RNAi on planta (Continuous exposure) – second instar nymph

Jack soy bean seeds were grown until reaching V2-V3 development stage as previously described. The plants were sprayed with a 15 % sucrose solution containing 75U/ml bacterial lysate expressing non targeting GFP or target dsRNA. For each condition a total volume of 2 ml was sprayed on the plant and replicated 3 times.

- 15 Twenty five second instar nymphs were placed on each sprayed plant and placed in a 2 compartment box (Fig.19) coated with Fluon PTFE (Blades Biological Ltd) to prevent nymphal escape. Spraying of the plants, rearing of insects and storage of the plants was performed as previously described. Pictures were taken daily to capture plant protection and nymphal mortality was recorded on day 3, 10 and 14 days post exposure to the AI.

- 20 Low feeding damage by nymphs of the soy bean plant and high mortality of nymphs was observed when plants were sprayed with a solution of 15% sucrose containing bacteria expressing dsRNA of lethal target dsRNA when compared to the damage incurred on a soybean plant and mortality of nymphs when plants were sprayed with a solution of 15% sucrose bacterial lysate expressing non target GFP dsRNA (Fig.19 and Fig.20).

- 25 Differences in feeding damage were clearly evident 4 days post treatment. In both treatments background mortality during the first 3 days post application was about 30%. A lethal RNAi effect on plants sprayed with 15% sucrose containing bacteria expressing dsRNA of lethal target dsRNA started to appear 7 days post application.

RNAi on planta (Continuous exposure) – adults

Jack soy bean seeds were grown until reaching V3 development stage as previously described. The plants were sprayed with a 15 % sucrose solution containing 75U/ml bacterial lysate expressing non targeting GFP dsRNA or target dsRNA. For each condition a total volume of 2 ml was sprayed on one plant and replicated 3 times.

5

Ten adults were placed on each sprayed plant and each plant was placed in an insect cage.

Mortality was recorded on day 3, 6, 10 and 14 days post exposure to the AI.

Spraying of the plants, rearing of insects and storage of the plants was performed as previously described.

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High mortality of adults was observed when plants were sprayed with a suspension of 15% sucrose and bacteria expressing dsRNA of a lethal target compared to the mortality of adults on plants sprayed with GFP dsRNA (Fig 21). A lethal RNAi effect started to appear 7 days post application.

15

RNAi on planta (Continuous exposure) – Mix nymphal stages/semi field

Jack soy bean seeds were grown until reaching V3 development stage as previously described.

Plants were sprayed with a 15 % sucrose solution containing 75U/ml bacterial lysate expressing

20

non targeting GFP dsRNA or target dsRNA. For each condition a total volume of 2 ml was sprayed on one plant. Each condition was replicated 12 times. The 12 plants were placed in 1 insect cage and second, third, fourth and fifth instar nymphs (8 each) were placed in the insect cage.

25

Pictures were taken on a daily basis to capture plant protection and mortality was recorded on day 3, 6, 10, 14 and 16 days post exposure to the AI.

Spraying of the plants, rearing of insects and storage of the plants was performed as previously described.

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Plants sprayed with a suspension of 15% sucrose and bacteria expressing dsRNA of a lethal target induced high mortality of nymphs (Fig. 22), impeded development into adulthood and nymphs induced less feeding damage to the soy bean plants compared to plants sprayed with non-target dsRNA (Fig.23). Differences in feeding damage were clearly evident 6 days post treatment.

A lethal RNAi effect on plants sprayed with 15% sucrose containing bacteria expressing dsRNA of lethal target dsRNA started to appear 7 days post application.

- 5 Fig.1 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal target naked dsRNA in an in vitro assay in the absence of a carbohydrate and in the presence of 15% sucrose. The RNAi response of nymphs feeding on the target dsRNA in the presence of sucrose is visible in the second week post feeding. No RNAi response is observed when feeding on target dsRNA in the absence of a carbohydrate.
- 10 Fig.2 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal target naked dsRNA in an in vitro assay in the presence of 1% and 15% sucrose. The RNAi response of nymphs when feeding on the target dsRNA in the presence of sucrose is visible in the second week post feeding.
- Fig.3 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal
15 target naked dsRNA in an in vitro assay in the presence of 5% and 10% glucose. The RNAi response of nymphs when feeding on the target dsRNA in the presence of glucose is visible in the second week post feeding.
- Fig.4 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal
20 target naked dsRNA in an in vitro assay in the presence of 5% and 10% fructose. The RNAi response of nymphs when feeding on target dsRNA in the presence of fructose is visible in the second week post feeding.
- Fig.5 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal
25 target naked dsRNA in an in vitro assay in the presence of 1% and 15% maltose. The RNAi response of nymphs when feeding on target dsRNA in the presence of maltose is visible in the second week post feeding.
- Fig.6 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal target naked dsRNA in an in vitro assay in the presence of 5% and 10% raffinose. The RNAi response of nymphs when feeding on target dsRNA in the presence of raffinose is visible in the second week post feeding.

Fig.7 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal target naked dsRNA in an in vitro assay in the presence of 1% and 5% mannitol. The decrease in mortality of nymphs during the first week of feeding on non-target dsRNA indicates that at the given concentrations the mannitol is toxic.

- 5 Fig.8 shows the mortality of second instar nymphs after feeding on non-target (GFP) and lethal target naked dsRNA in an in vitro assay in the presence of 15% and 1% sorbitol. The decrease in mortality of nymphs during the first week of feeding on non-target dsRNA indicates that at the given concentrations the sorbitol is toxic.

- 10 Fig. 9 shows the mortality of second instar nymphs after feeding on bacterial lysate expressing non-target (GFP) and lethal target dsRNA in the presence of 15% sucrose. The RNAi response of nymphs when feeding on target dsRNA in the presence of sucrose is visible in the second week post feeding.

- 15 Fig. 10 shows the mortality of second instar nymphs after feeding on bacterial lysate expressing non-target (GFP) and lethal target dsRNA in the presence of 5% sucrose. The RNAi response of nymphs when feeding on target dsRNA in the presence of sucrose is visible in the second week post feeding.

- 20 Fig. 11 shows the mortality of second instar nymphs after feeding on bacterial lysate expressing non-target (GFP) and lethal target dsRNA in the presence of 15% maltose. The RNAi response of nymphs when feeding on target dsRNA in the presence of sucrose is visible in the second week post feeding.

- Fig. 12 shows the mortality of second instar nymphs after feeding on bacterial lysate expressing non-target (GFP) and lethal target dsRNA in the presence of 5% maltose. The RNAi response of nymphs when feeding on target dsRNA in the presence of sucrose is visible in the second week post feeding.

- 25 Fig. 13 shows the mortality of second instar nymphs after feeding on bacterial lysate expressing non-target (GFP) and lethal target dsRNA in the presence of 15% lactose. No RNAi response of nymphs when feeding on target dsRNA in the presence of lactose is visible in the second week post feeding.

- 30 Fig. 14 shows the mortality of second instar nymphs after feeding on bacterial lysate expressing non-target (GFP) and lethal target dsRNA in the presence of 5% lactose. No RNAi response of

nymphs when feeding on target dsRNA in the presence of lactose is visible in the second week post feeding.

Fig. 15 shows high uptake of FITC fluorescent dye nymphs on V1-V2 plants sprayed with a suspension of 75U/ml of bacterially expressed GFP dsRNA in 15% sucrose and very low uptake of FITC fluorescent dye in absence of 15% sucrose.

Fig. 16 shows low survival rate of nymphs on V2-V3 soybean plants sprayed with a suspension of 75U/ml bacterial lysate expressing GFP dsRNA and high survival rate on plants sprayed with a suspension of 75U/ml bacterial lysate expressing GFP dsRNA in the presence of sucrose.

Fig. 17 shows low survival rate of a first species of Stink Bug nymphs when exposed for 3d to plants sprayed with a 15% sucrose suspension of 75U/ml bacterial lysate expressing target dsRNA compared to the negative control (GFP).

Fig. 18 shows low survival rate of a second species of Stink Bug nymphs when exposed for 3 days to plants sprayed with a 15% sucrose suspension of 75U/ml bacterial lysate expressing lethal target dsRNA compared to the negative control (GFP).

Fig. 19 shows plant protection of the soy bean plant when the plants were sprayed with a solution of 15% sucrose containing bacterial lysate expressing dsRNA of a lethal target dsRNA compared to the damage incurred on the control plants (sprayed with GFP dsRNA).

Fig. 20 shows high mortality of nymphs continuously exposed to V2-V3 soy bean plants sprayed with a suspension of 75U/ml bacterial lysate expressing lethal target dsRNA compared to the mortality of nymphs exposed to control (GFP) bacterial lysate.

Fig. 21 shows high mortality rate of adults on V3 soybean plants sprayed with a 15% sucrose solution containing 75U/ml bacterial lysate expressing target dsRNA compared to low mortality of adults on V3 soybean plants sprayed with a suspension of 75U/ml bacterial lysate expressing GFP dsRNA

Fig. 22 shows high mortality of all Stink bug nymphal stages continuously exposed to 12 V3 soy bean plants sprayed with a solution of 15% sucrose containing 75U/ml bacterial lysate expressing lethal target dsRNA compared to the mortality of nymphs exposed to control (GFP) bacterial lysate.

Fig. 23 shows plant protection 16 days post infestation with nymphs (all stages), when the plants were sprayed with a solution of 15% sucrose containing bacterial lysate expressing dsRNA of a lethal target dsRNA (Target dsRNA) compared to the damage incurred on the control plants (Non target dsRNA).

CLAIMS

1. A pesticidal composition comprising a double-stranded ribonucleic acid (dsRNA) and a carbohydrate, wherein said dsRNA is capable of RNAi gene silencing and comprises a strand that is complementary to at least part of a nucleotide sequence of a gene from a plant pest organism.
2. The pesticidal composition of claim 1, wherein the composition is substantially devoid of salts
3. The pesticidal composition of claims 1 or 2, further comprising a binding agent and/or an anti-desiccant and/or a humectant and/or rheology modifier and/or a bulking agent.
4. The pesticidal composition according to any of the preceding claims wherein said dsRNA is synthetically transcribed in vitro.
5. The pesticidal composition according to claims 1, 2 or 3 wherein said dsRNA is transcribed by a microorganism.
6. The pesticidal composition of claim 5, wherein said microorganism is inactivated.
7. The pesticidal composition according to claim 6, wherein the dsRNA in the composition is comprised in an intact microbial cell or a cell lysate.
8. The pesticidal composition of claims 5, 6 or 7 wherein said microorganism is selected from the group of bacteria, fungi, algae and yeast.
9. The pesticidal composition according to any of the preceding claims, wherein said carbohydrate is provided at a concentration that is sufficient to promote said plant pest organism to take up dsRNA to enable effective RNAi gene silencing.
10. The pesticidal composition of claim 9, wherein said carbohydrate is present in the composition at an amount of from 0.01 % to 20% of total volume of the composition.
11. The pesticidal composition according to any preceding claim wherein said carbohydrate comprises at least one glucose residue and/or at least one fructose residue.
12. The pesticidal composition according to claim 11, wherein said carbohydrate is selected from the group consisting of sucrose, maltose, glucose, fructose and raffinose.

13. The pesticidal composition according to claim 1 to 10, wherein said carbohydrate is a sugar alcohol.
14. The pesticidal composition according to any of the preceding claims, wherein said plant pest organism is of the order Hemiptera.
15. The pesticidal composition according to claim 14, wherein said Hemiptera plant pest is selected from the group consisting of green stink bug *Acrostemum hilare* (Say), *Brochymena quadripustulata* (Fabricius) rough stink bug, *Chlorochroa sayi* (Stål) Say stink bug, *Coptosoma xanthogramma* (White) black stink bug, *Euschistus servus* (Say) brown stink bug, *Euschistus tristigmus* (Say) dusky stink bug, *Euschistus variolarius* (Palisot de Beauvois) onespotted stink bug, *Halyomorpha halys* (Stål) brown marmorated stink bug, *Nezara viridula* (Linnaeus) southern green stink bug, *Oebalus pugnax* (Fabricius) rice stink bug, *Perillus bioculatus* (Fabricius) twospotted stink bug, *Piezodorus guildinii* (Westwood) redbanded stink bug, *Plautia stali* Scott oriental stink bug, *Thyanta custator accerra* McAteer shouldered stink bug, *Euschistus heros* Neotropical brown stink bug, *Dichelops melacanthus*.
16. The pesticidal composition according to claims 1 to 15, further comprising at least one other pesticidally active ingredient, wherein said pesticidally active ingredient targets the same plant pest organism.
17. The pesticidal composition of claims 1 to 15, further comprising at least one other pesticidally active ingredient, wherein said pesticidally active ingredient targets a different plant pest organism.
18. A method for controlling a plant pest organism comprising applying to the plant, to the seed of a plant, or locus of a plant or seed the composition of any of the preceding claims.
19. The method according to claim 18, wherein said composition is ingested by said plant pest organism.
20. A method for controlling a plant pest organism comprising applying to a plant, sequentially and in any order, a dsRNA capable of RNAi gene silencing comprising a strand that is complementary to at least part of a nucleotide sequence of a gene from a

plant pest organism, a carbohydrate according to any of the preceding claims and optionally another pesticidally active ingredient.

21. The use of the composition according to claims 1 to 17 for controlling plant pest growth and/or infestation.
22. The use of a carbohydrate, in a composition comprising pesticidal dsRNA, to satiate the appetite of a pest so that the production of salivary nucleases by the pest is substantially inhibited.
23. A method of substantially inhibiting the production of salivary nucleases in a pest fed a composition comprising a pesticidal dsRNA by including a carbohydrate in the composition.
24. The use or method of claim 22 and 23 wherein the carbohydrate is sucrose, the pest is a plant pest, the plant is a soybean plant, the pest is a stink bug and the carbohydrate is present in the composition at an amount of 0.01% to 20% of the total volume of the composition.

Figures

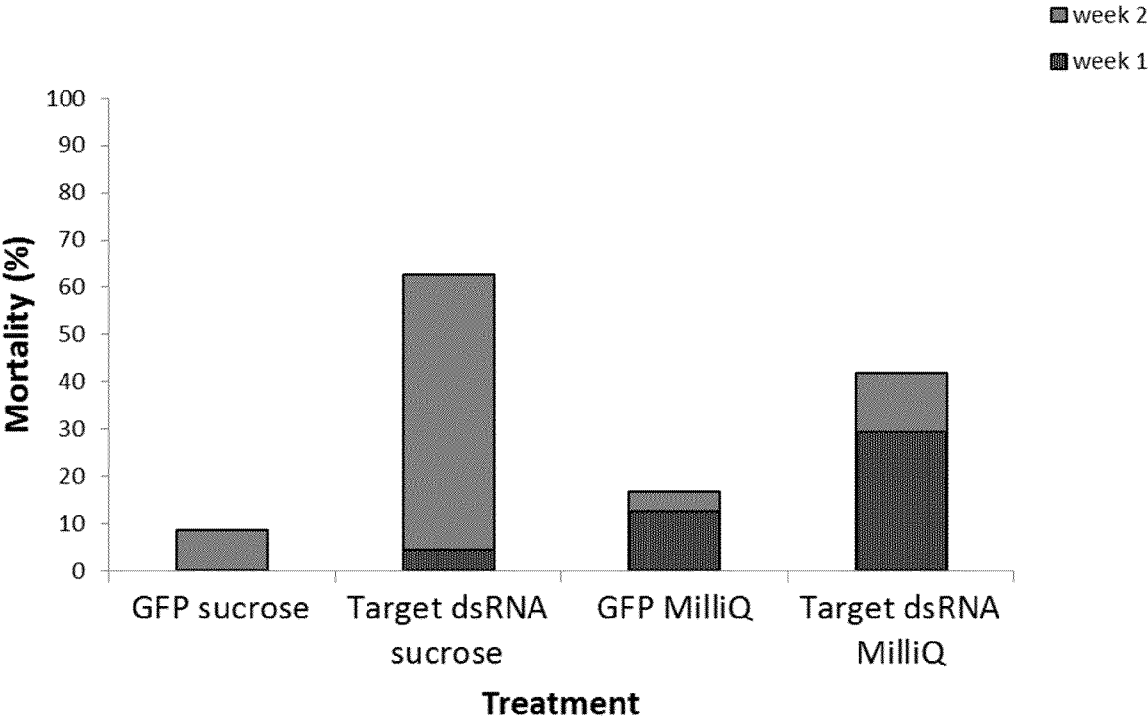


Figure 1

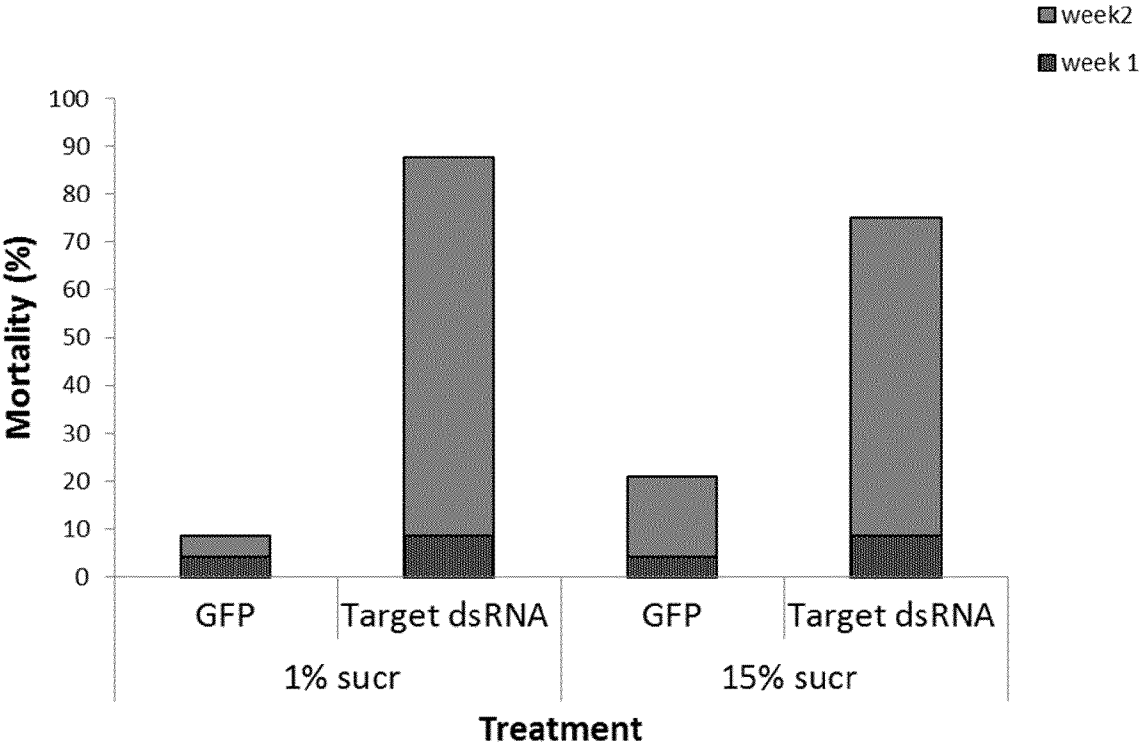


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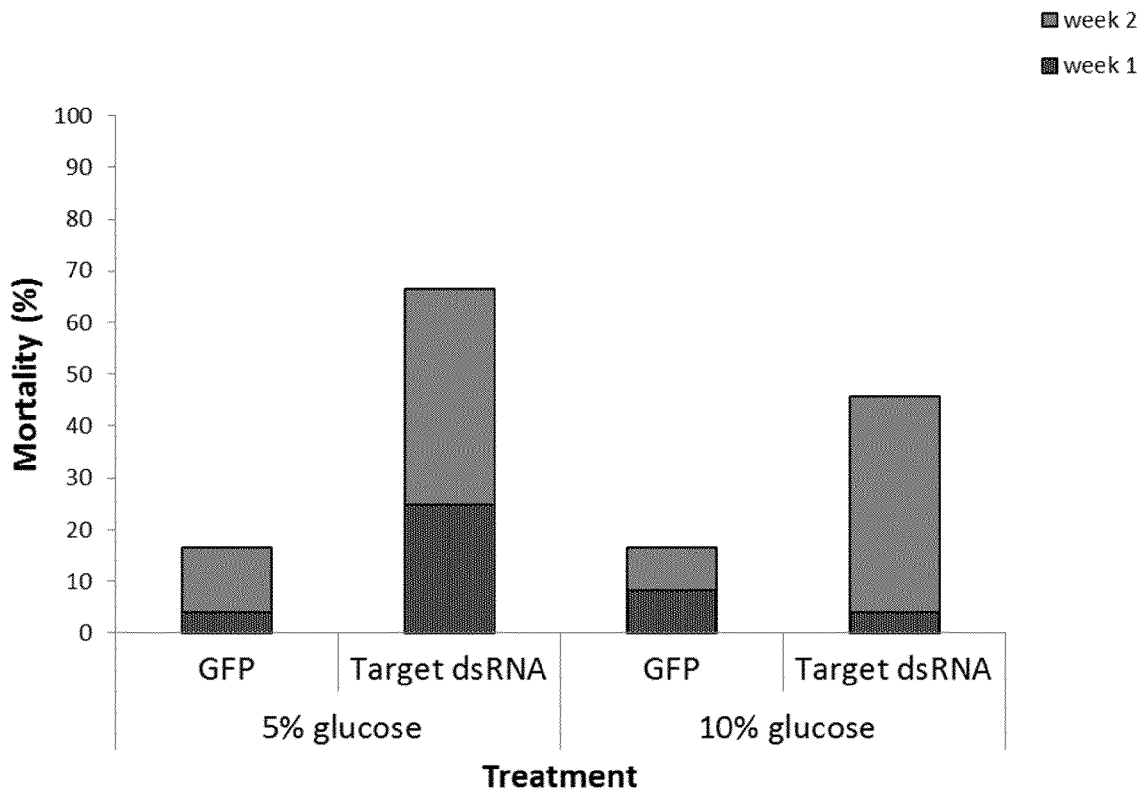


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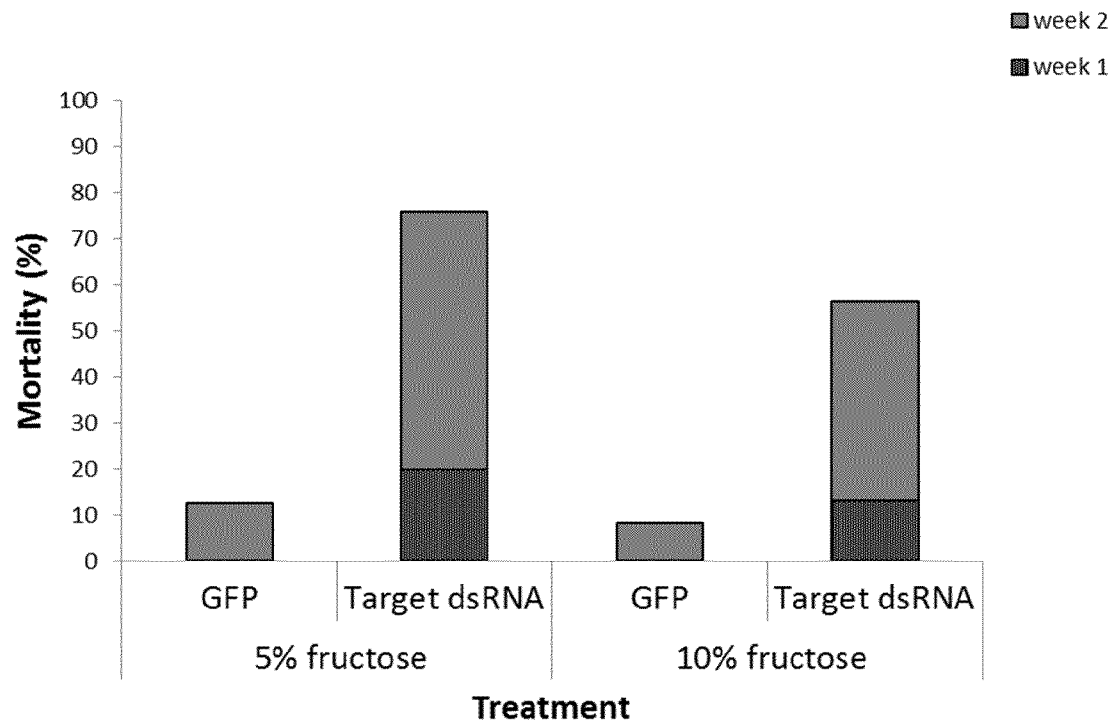


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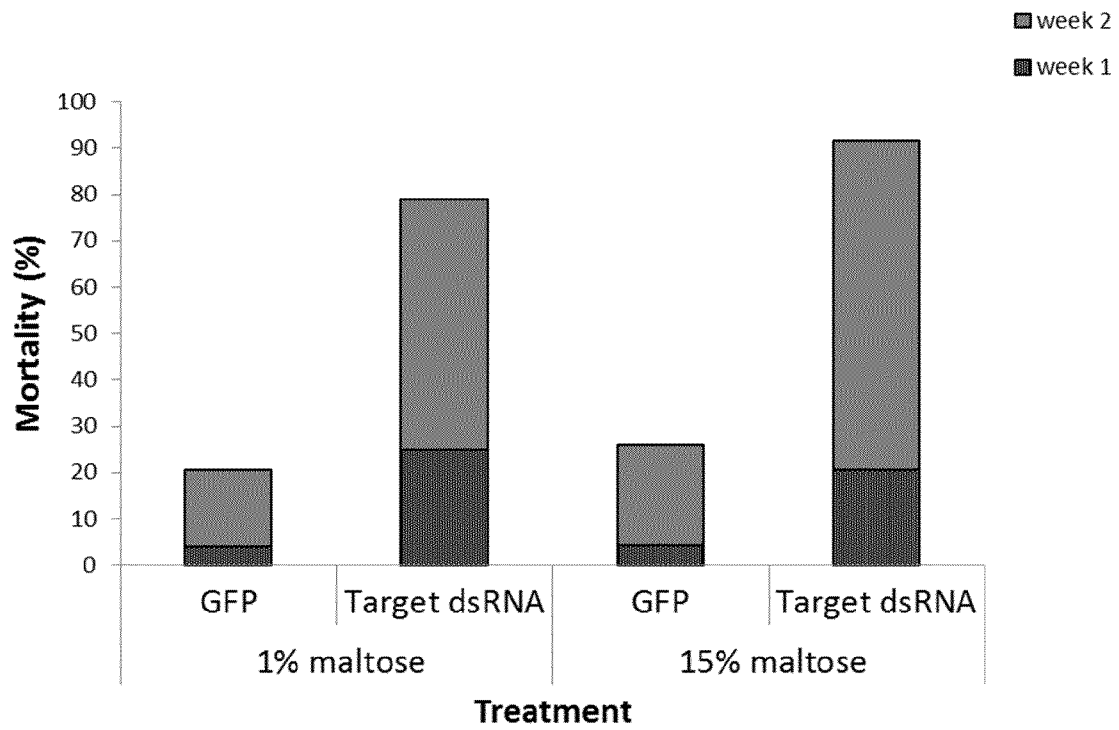


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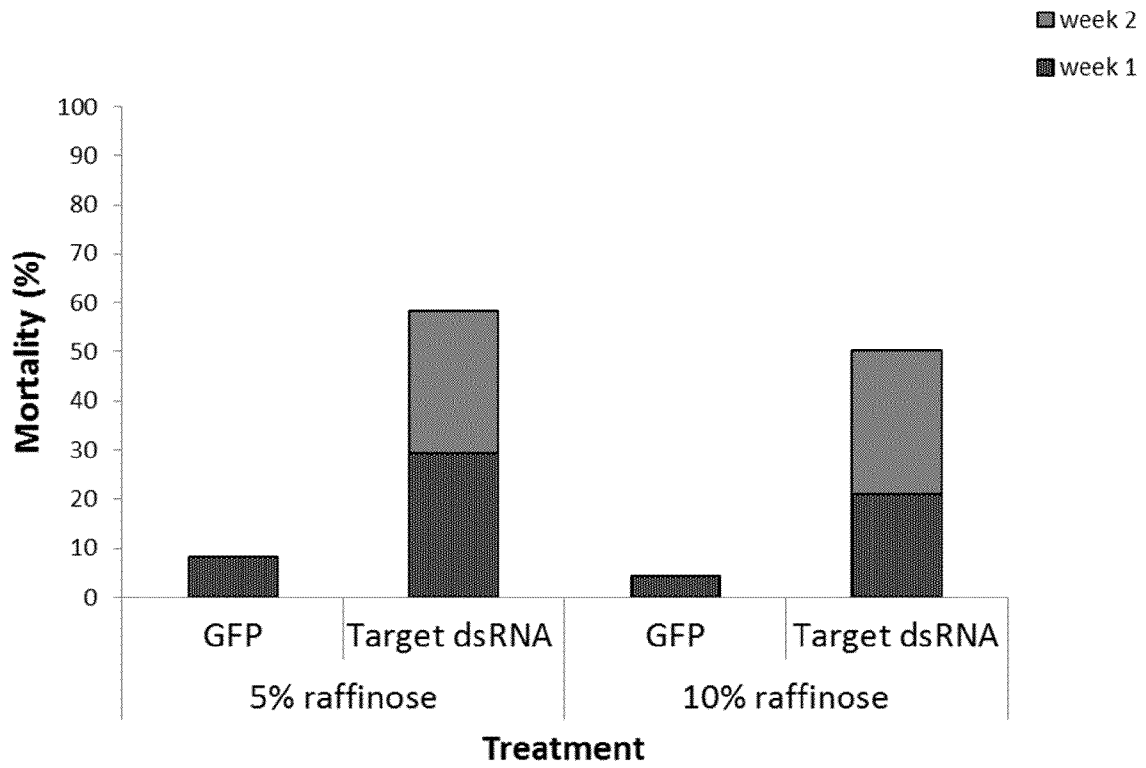


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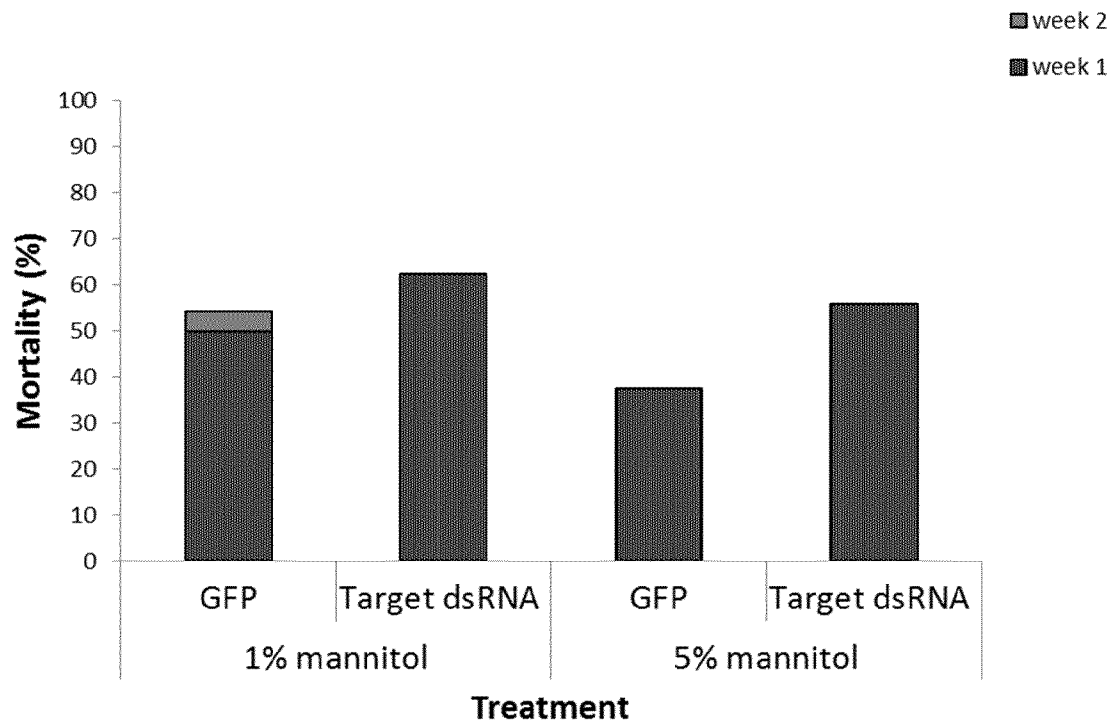


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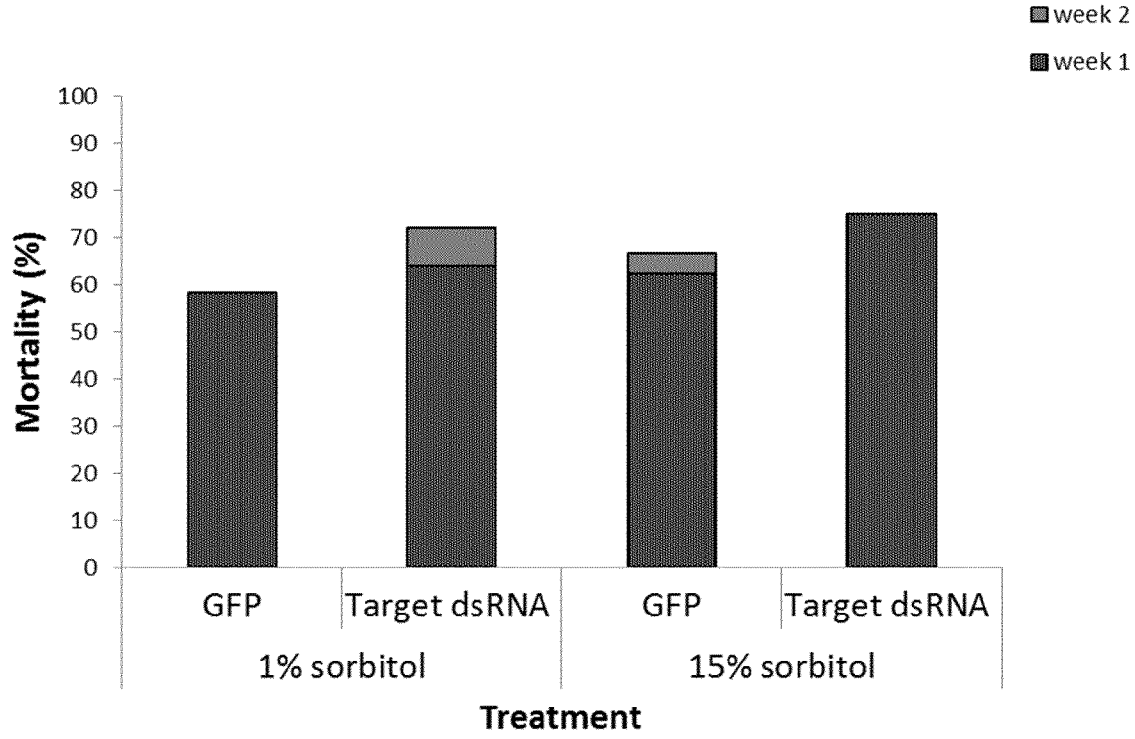


Figure 8

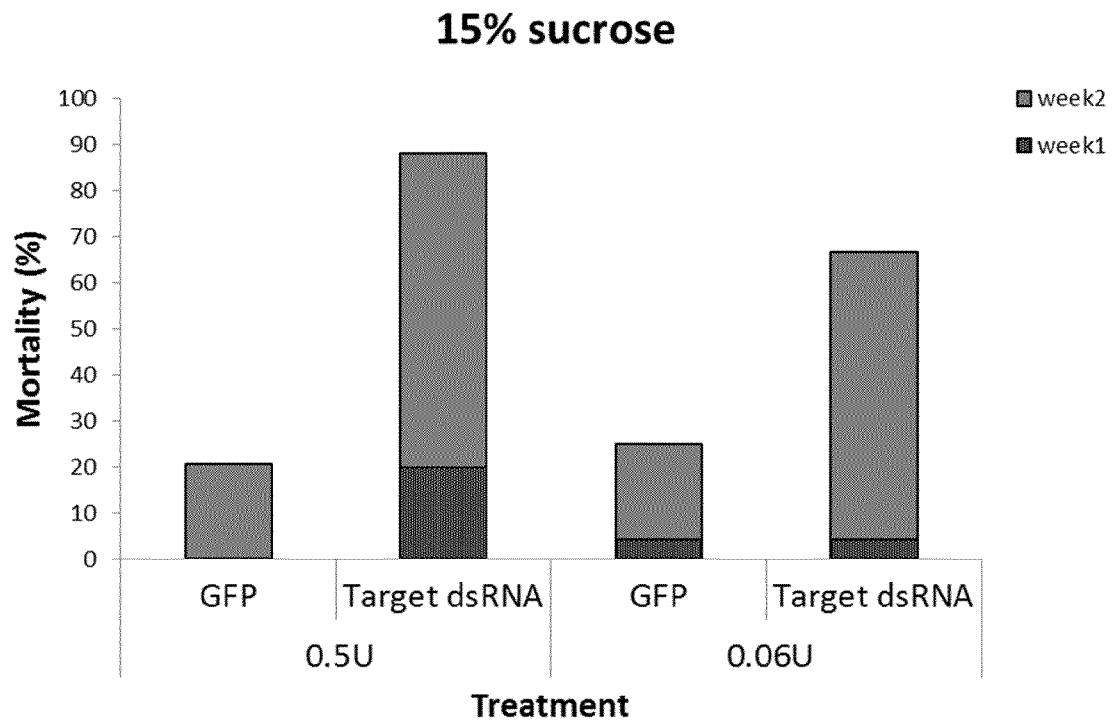


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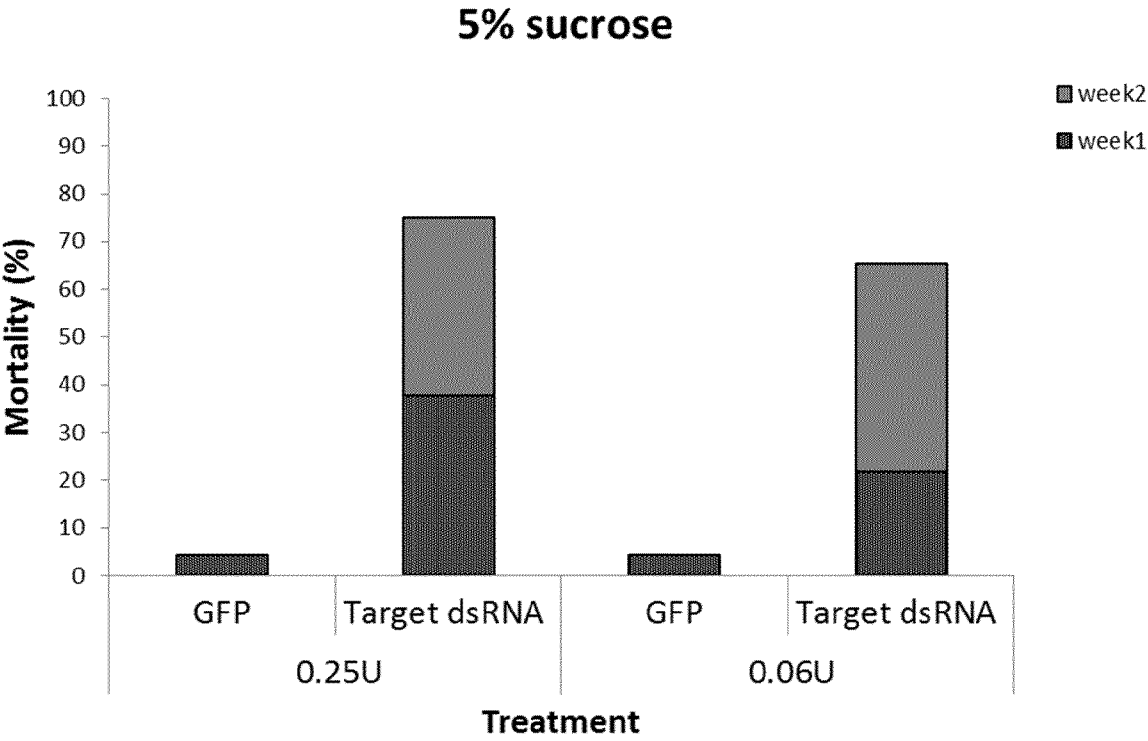


Figure 10

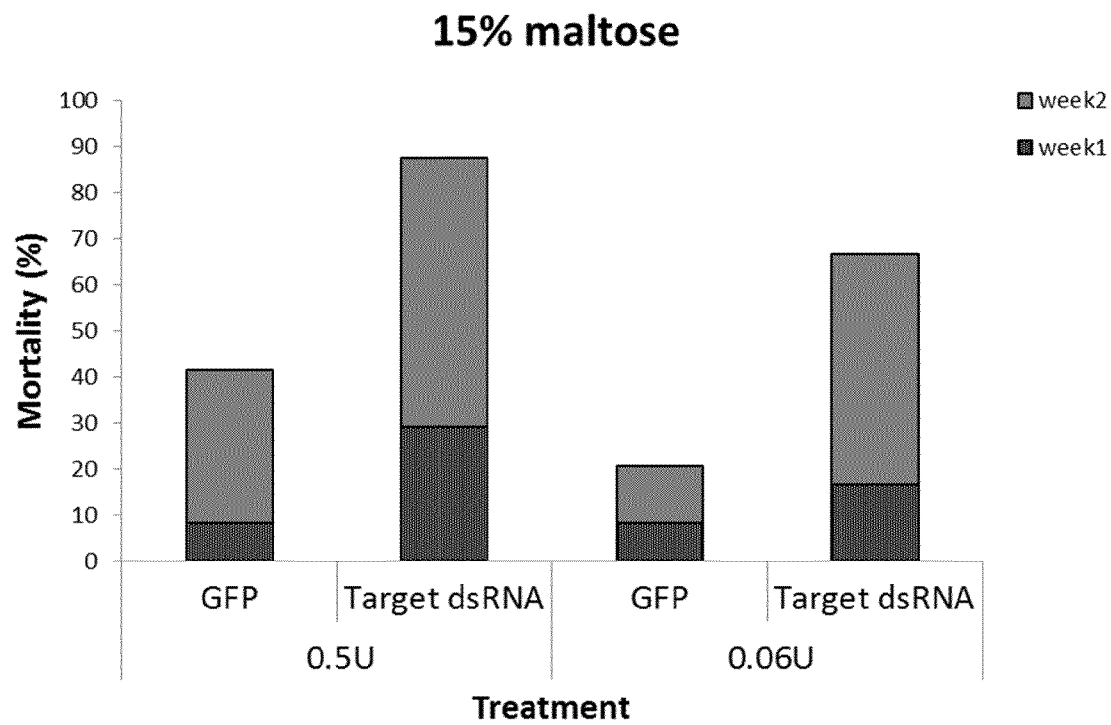


Figure 11

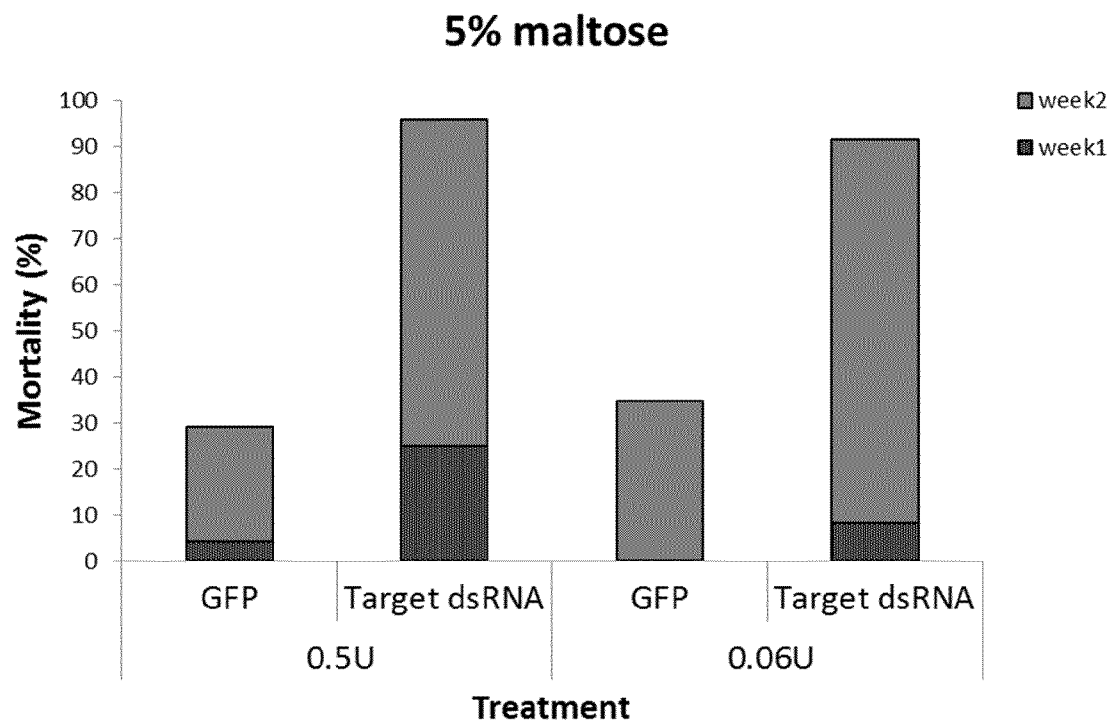


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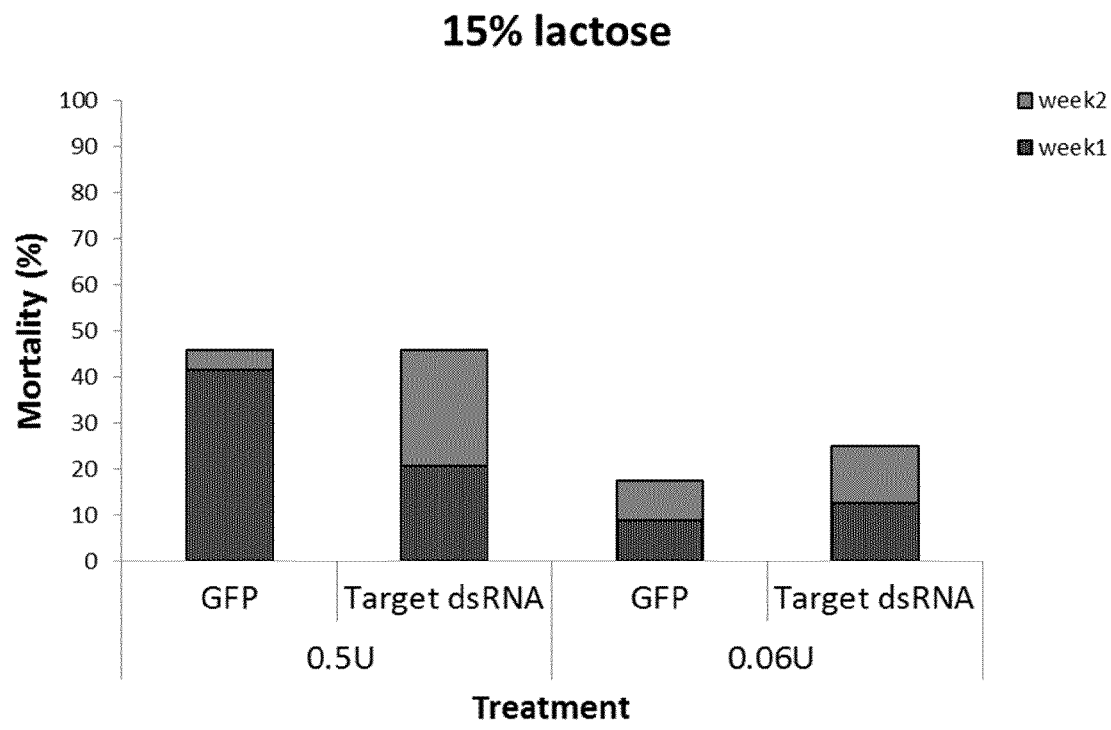


Figure 13

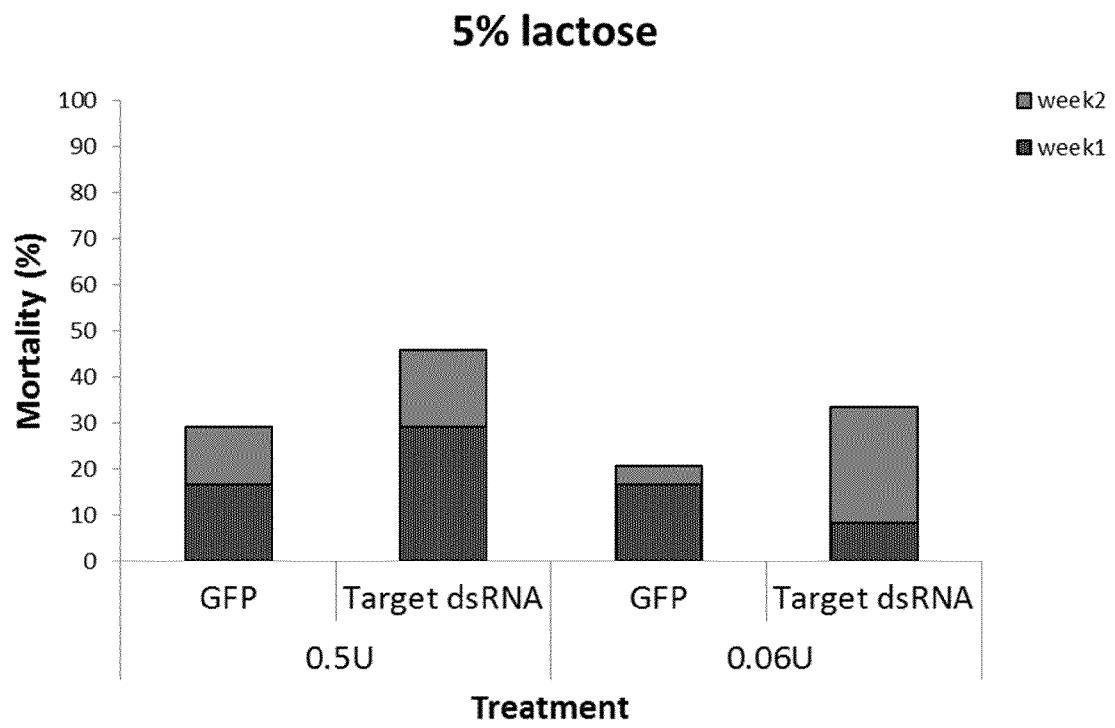


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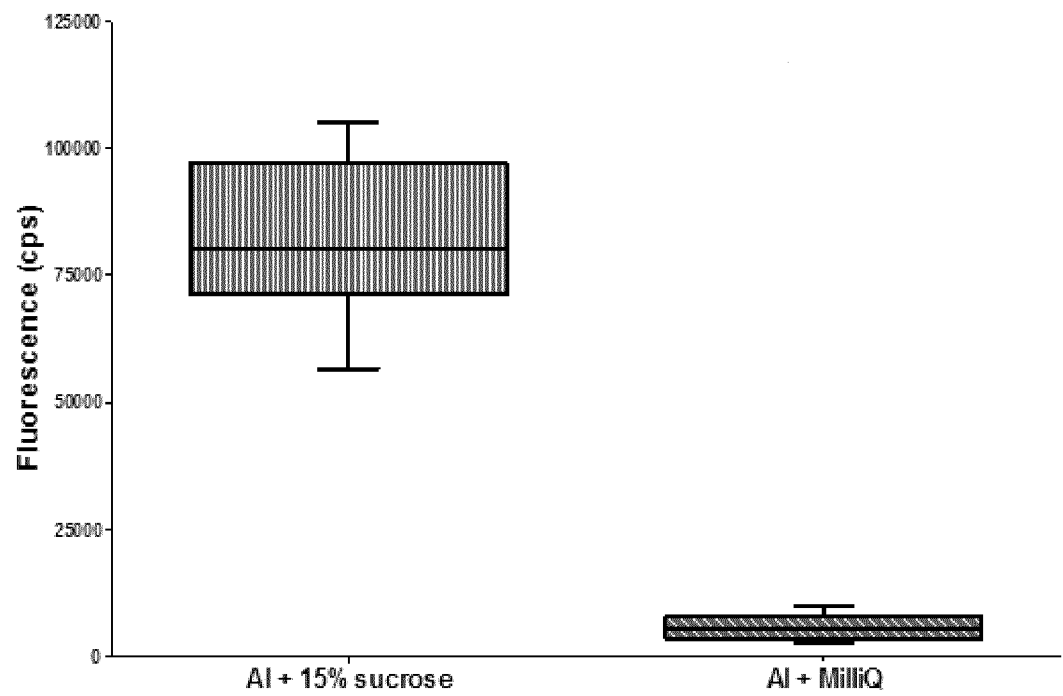


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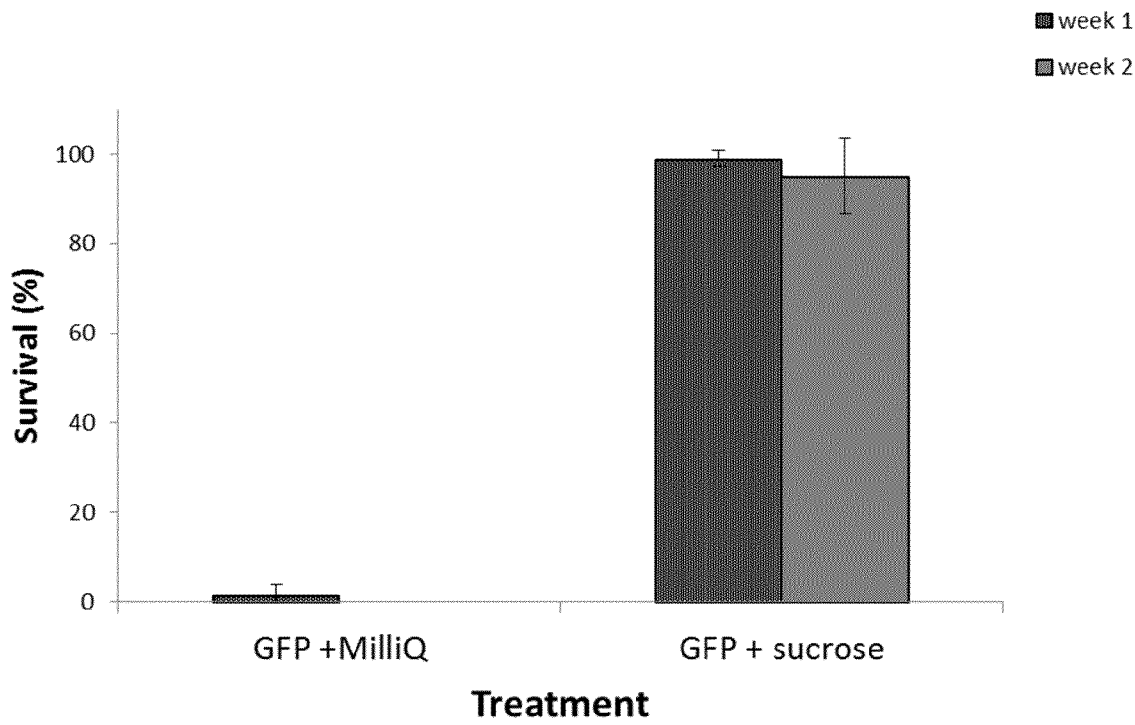
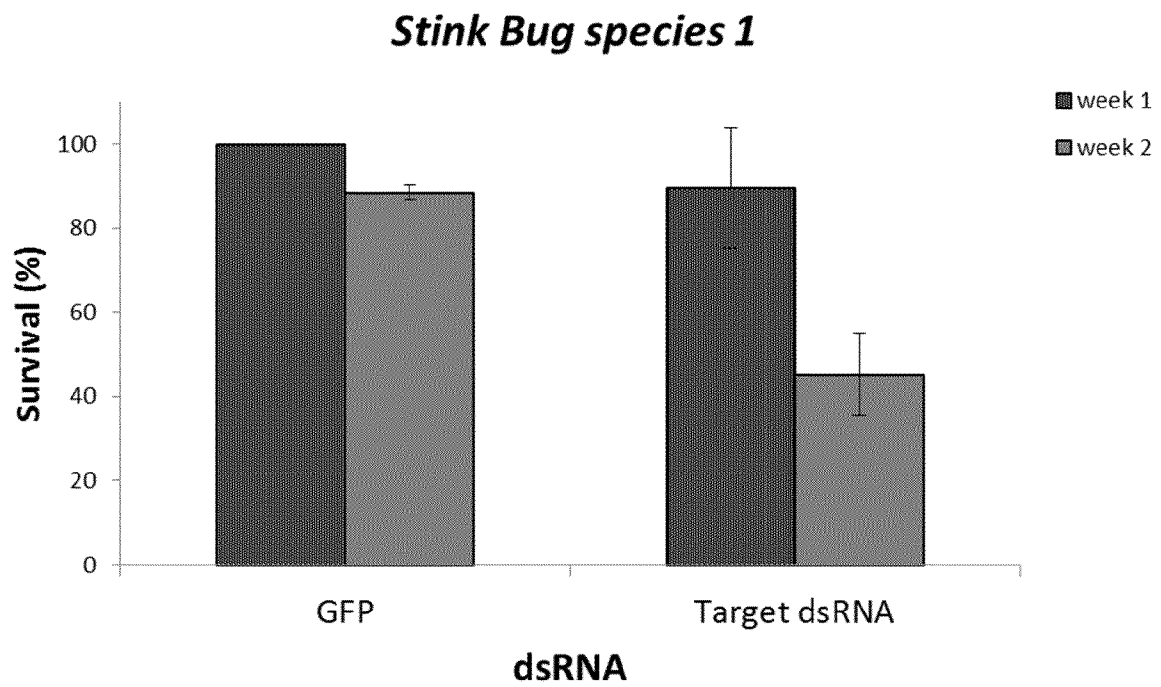
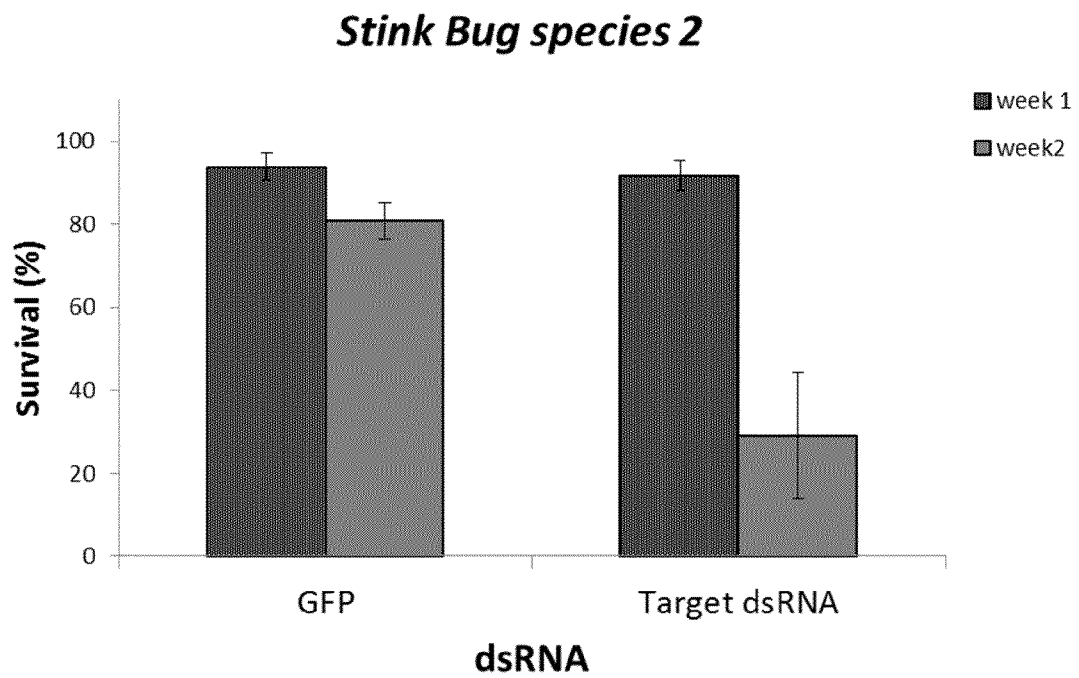


Figure 16

**Figure 17**

**Figure 18**

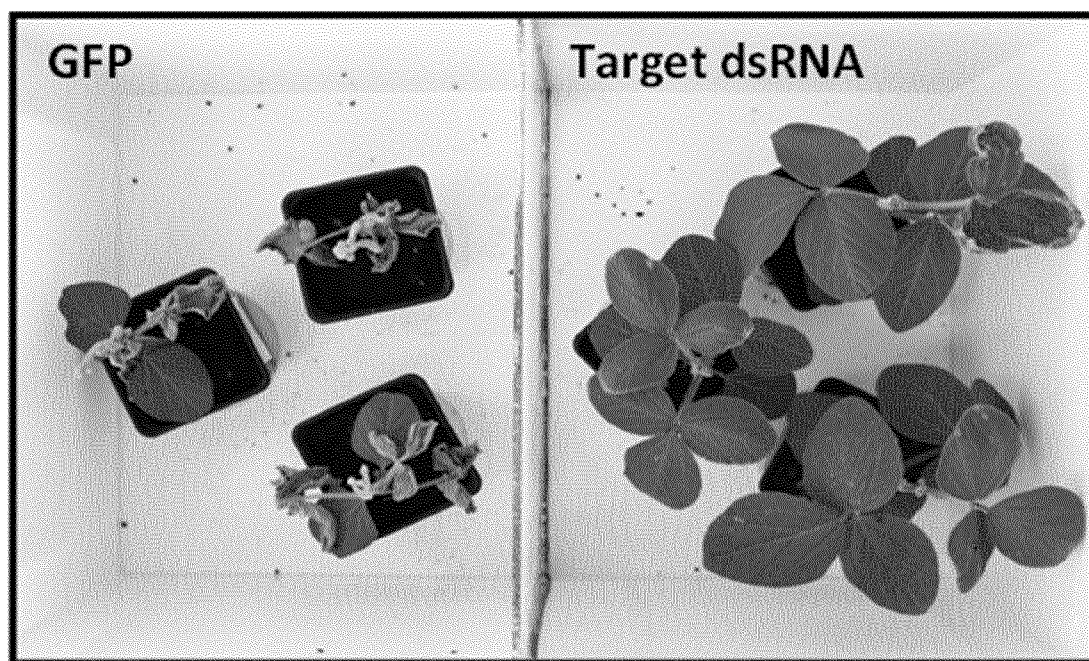


Figure 19

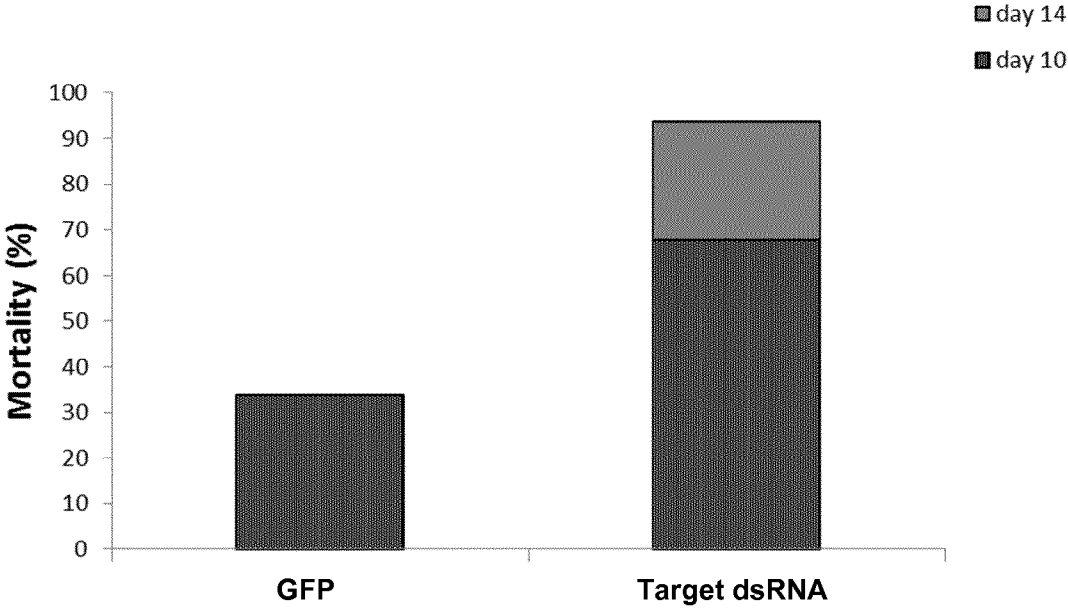


Figure 20

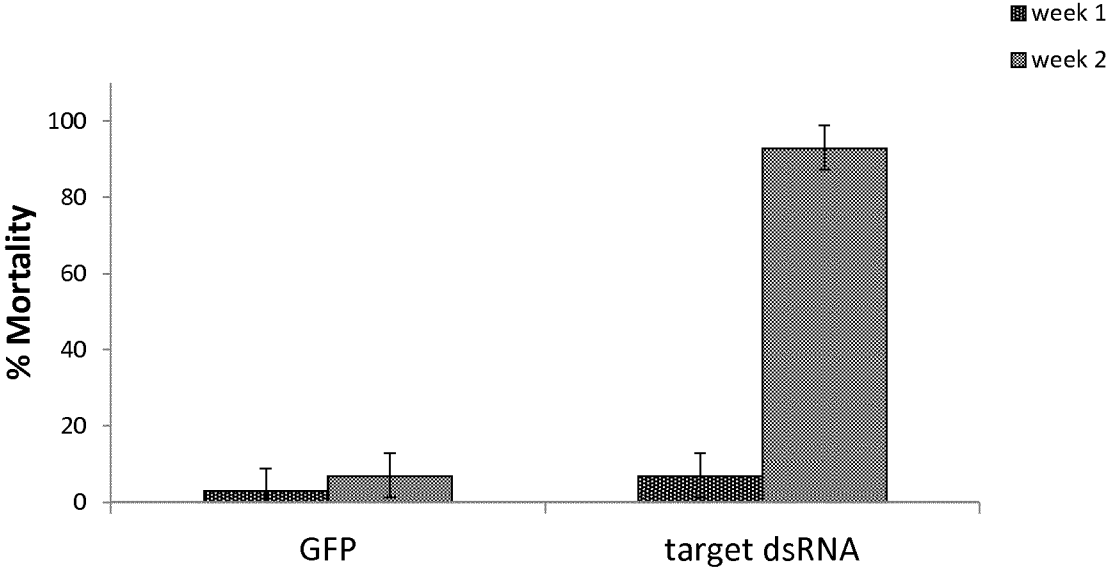


Figure 21

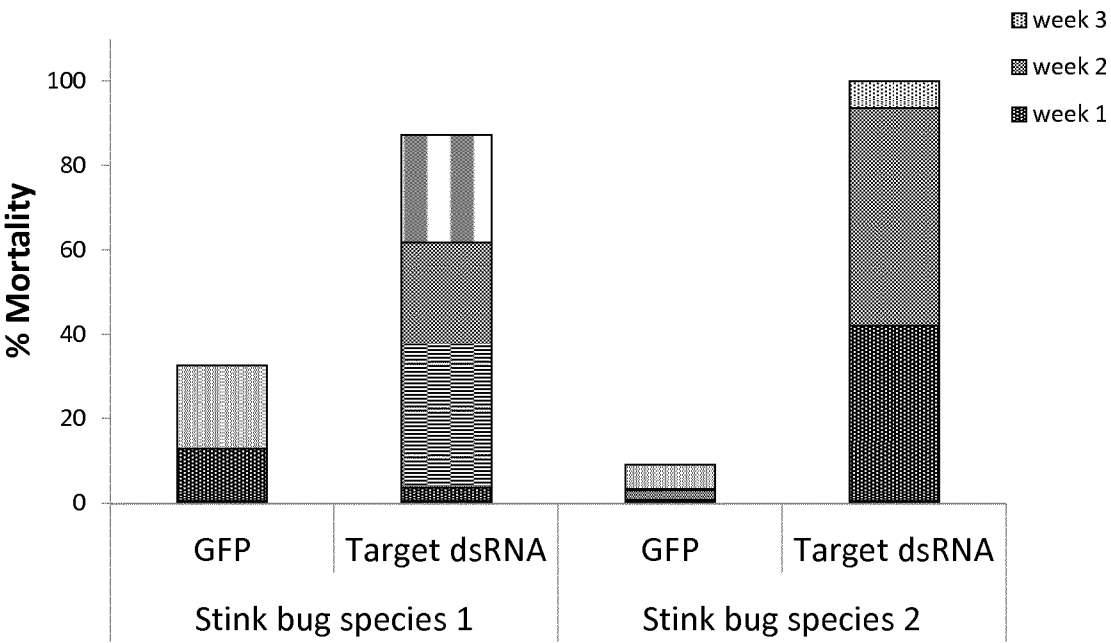


Figure 22



Target dsRNA

Non Target dsRNA

Figure 23