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Laboratory sprayer for dsRNA application: Design and bioassay validation

Triin Kallavus[a,b,*], Riina Kaasik[a], Tõnu Leemet[c], Kaarel Soots[c], Liina Soonvald[a], Silva Sulg[a], Eve Veromann[a]

[a] Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu 51006, Estonia
[b] Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent 9000, Belgium
[c] Institute of Forestry and Engineering, Estonian University of Life Sciences, Tartu 51006, Estonia

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ABSTRACT

The shortage of commercially available and reliable laboratory spraying equipment for testing different preparations can be a major obstacle to achieve field-comparable results in the laboratory conditions.
RNA interference is natural biological process which, when used for plant protection, can be designed method combining sustainability and minimal environmental side effects. Spraying of dsRNA is a field-relevant method that should ensure consistency and repeatability if conducted in laboratory.
We built a portable spray device for laboratory use and tested its suitability for dsRNA application. For that, we carried out bioassay on three plant species with different leaf surface textures. DsRNA were detected in all samples 3 days post-treatment indicating its suitability for dsRNA delivery.
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- Bioassay on three plant species with different leaf surface textures.

DsRNA were detected in all samples 3 days post-treatment indicating its suitability for dsRNA delivery.

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* Corresponding author at: Chair of Plant Health, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu 51006, Estonia.
E-mail address: triin.kallavus@emu.ee (T. Kallavus).

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Resource availability:	• https://support.stratasys.com/en/printers/fdm-legacy/uprint • https://en.dmgmori.com/products/machines/milling/vertical-milling/m/m1 • *https://proplastik.ee/* • https://www.iwata-airbrush.com/iwata-power-jet-lite.html • https://www.iwata-airbrush.com/revolution-trigger-brush.html • https://genolution.co.kr/ • https://www.qiagen.com/mg • https://www.thermofisher.com/ee/en/home/brands/invitrogen.html • https://www.eppendorf.com/us-en/eShop-Products/PCR/Thermocyclers/Mastercycler-nexus-p-PF-14698 • https://cran.r-project.org/bin/windows/base/old/3.6.1/

Method details

Introduction

Insect pests are one of the mayor cause of yields losses and their management relies commonly on synthetic insecticides applied by spraying the plants. One way to decrease pre- and post-harvest losses of agricultural crops by targeting specific pests while minimizing negative effects on non-target species is the application of RNA interference (RNAi) [1–3]. RNAi based solution in plant protection is potentially pest specific, which leads to reduced risk on non-target organisms [4] but a vital part is the delivery and sufficient uptake of double-stranded RNA (dsRNA) by the pest. RNAi have been successfully implemented through environmental dsRNA on multiple plant pathogens [5–7] and insects pests [1,8–10]. Direct spray application of dsRNA leading to spray induced gene silencing (SIGS) [11] has an advantage of being more environmentally acceptable and GMO-free compared to host induced gene silencing (HIGS) [12].

While several studies have concentrated on the stability of exogenous dsRNA, which is the basis of the successful RNAi [13,14,8], a plant surface texture can also affect the effectiveness of the treatment. Spraying plants can be challenging as leaf surfaces can either be waxy, hairy, smooth, or warty making the plant hydrophobic and may withhold the equal spreading or uptake of the treatment solution.

Standard spraying equipment can be used in laboratory studies such as Potter spray tower [14] or spray flask [5]. The disadvantages of the Potter spray while targeting the whole plant is its stationarity and unconvertible spraying height while spray flasks could not capacitate consistency. While both, the concentration and exposure of dsRNA can affect the uptake and survival of the pest, it is necessary to assure uniform treatment in laboratory studies. To have steady and continuous control of the applied dose along with uniform coverage we built portable spray device with treatment chamber extracting the knowledge from experimental apparatus originally aimed for applying entomopathogenic fungi [15–17].

Here, we describe standardized and repeatable bioassay approach for studying SIGS on plant experiments. The bioassay approach was validated using green fluorescent protein (GFP)-marked double-stranded RNA (dsRNA-GFP) on three different plant species that were chosen by their different surface texture.

Methods

Design and fabrication of the experimental spray tower/device

The spray tower was built in the Estonian University of Life Sciences (Tartu, Estonia) based on the designs by Erdos et al. [16]; Moura Mascarin et al. [15] and Spence et al. [17].

In general, a hybrid fabrication approach was taken towards building the custom hardware components of the spray tower. The lower support component for the plastic cylinder and the three-sided bracket at the top were made utilizing the additive manufacturing technology i.e. 3D printing. Cylinder cover and support pillars were made by the subtractive manufacturing methods. The top cover was milled, and the pillars were turned and tapped on the CNC machining centers. The 200 mm diameter acrylic (PMMA) cylinder with the wall thickness of 2 mm was a commercial product (Proplastik OÜ). Standard M5 bolts and nuts from stainless steel were used for fastening the individual part.

3D printed components: bottom support and three sided bracket

Given the relatively large diameter of the cylinder, the support was designed as two separate parts. This is directly driven by the size of the printing area of the Stratasys uPrint SE Plus 3D printer available for the project. At the same time, this adds simplicity and speed to the process when another cylinder has to be placed in the device. Given the relative tallness of the cylinder, a set of pads was designed, and 3D printed, to be attached to the bottom of the cylinder, increasing the contact area and static stability of the device (Fig. 1). Two parts of the support click together mechanically, and the pads were locked in place by three M5 bolts. The

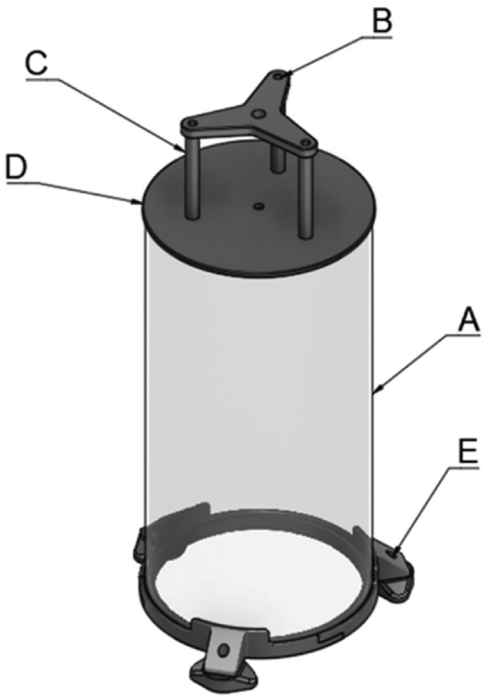

Fig. 1. Overall design of the spray tower system with the component listing: A – cylinder; B – three-sided bracket; C – support pillars; D – top cover; E – bottom support component (T. Leemet).

printed parts had layer height of 0.254 mm. Automated support features from Stratasys SR-30 (soluble) material were added while printing and removed mechanically afterwards.

Machined components: top cover and support pillars

Because of the overall size of the top cover, CNC milling was used to manufacture this part. Its geometry made it possible to carry out all machining in one setup on the 3-axis vertical machining center DMG MORI M1.

Support pillars connect the three-sided bracket to the top cover. Three of those were prepared from a 15 mm diameter aluminum alloy on a 2-axis CNC lathe HAAS SL-10. M5 standard thread holes are at both ends of the pillars for connecting the components. Inert fasteners from acid resistant stainless steel (A4-70) were chosen for the project. The three-sided bracket serves as a support to the spray gun from above while assembled on top of the cover. A gravity-fed Iwata Revolution HP-TR1 Side Feed Dual Action Trigger Airbrush with a 0.3 mm needle (screw-on type) is placed on top of an acrylic cylinder (width 200 mm, height 500 mm, thickness

Fig. 2. Gel electrophoresis s results of oilseed rape plant anthers (a) and buds (b), tomato and wheat plants (10, 15 or 20 mark pressure; "T" Tween 80, "S" Silvet 806 and "N" naked/pure, without surfactant).

2 mm) (Fig. 2). The airbrush is connected to a Iwata IS-925 Power Jet Lite compressor. The micro-sprayer can easily be disassembled for cleaning, disinfection or replacement of parts.

Spray tower adjustment

To identify the right combination of spraying height, pressure (PSI) and volume (µl) for bioassay, the different pressures and volumes were tested to cover evenly 70 mm diameter filter paper placed into a 90 mm diameter Petri dish at distances 24.5 and 12.5 cm from the spray gun. To evaluate the relationship between pressure (PSI) and volume (µl) to transfer efficiency, the filter papers were weighed before and after spraying. All pressure and volume combinations were replicated six times resulting in 54 observations. For better visual inspection of spray evenness, the same pressure and volume combinations were sprayed using distilled water and potassium permanganate solution (10 %) to 90 mm glass Petri disc (Fig. S1). All spray experiments were carried out at a room temperature (+21 ± 2 ˚C).

Bioassay

Bioassay validation was carried out on three plant species with different surface using green fluorescent protein (GFP) derived dsRNA (dsRNA-GFP) AgroRNA (Genolution, Seoul, South Korea) solution in water sprayed to the plants using previously described custom-made spray-tower. Plant species were selected based on their hydrophobicity, which is defined through water contact angle (WCA) [18–21] which for oilseed rape flowers is 155˚ [22], for tomato 80˚ [23] and wheat 130˚ [24].

Based on spray assessment all plant species were sprayed at 10, 15 and 20 PSI with 500 µl of solution at the height of 24,5 cm. Winter oilseed rape (*Brassica napus* L.) was treated at the yellow bud stage, tomatoes (*Solanum lycopersicum* L.) at the start of flowering stage and wheat (*Triticum aestivum* L.) plants at the heading stage. For treatment, 10 cm long stem were cut from the plant and placed separately inside spray cylinder. Spraying treatments were: 1 µl/ µg dsRNA-GFP with three surfactant options: pure (without surfactant), polysorbate 80 (Tween 80) or trisiloxane alkoxylate (Silwet 806), with nine replicas of each treatment and the experiment was replicated twice. After the treatment, stem cuttings were placed separately in water in growth chamber at 60 % relative humidity and 14:10 h light–dark cycle at +15 ˚C and +10 ˚C, respectively.

Plant material from all treatments were analyzed using PCR at day 4 post-treatment to estimate the efficacy of the custom-made spray-tower for dsRNA delivery and its stability on plants. RNA was extracted from 30 to 40 mg plant material, using RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's protocol. RNA concentration was quantified, and purity assessed, using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), with purity further verified via gel electrophoresis. The detection of dsRNA-GFP was performed from 300 ng of RNA, using a SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) with GFP primers (Forward-primer: CACATGAAGCAGCACGACTT, Reverse-primer TGCTCAGGTAGTGGTTGTCG). The PCR was performed on an Eppendorf Mastercycler (Hamburg, Germany) under the following conditions: 10 min at 75 ˚C, 30 min at 55 ˚C, 2 min at 94 ˚C, 40 cycles of 15 s at 94 ˚C, 30 s at 55 ˚C, 1 min at 68 ˚C, and 5 min at 68 ˚C. In order to denature the secondary structure of the dsRNA-GFP, a denaturing step of 10 min at 75 ˚C was added to the protocol. The PCR products were visualized on an agarose gel stained with ethidium bromide to confirm successful amplification. Positive and negative controls were run simultaneously throughout the experiment.

Statistical analysis

All analyses were conducted in R v3.6.1 (Team 2018). The relationships between transfer efficiency, volume and pressure were analyzed using a Pearson's correlation coefficient. Statistically significant differences were calculated using the analysis of covariance (ANCOVA in PROC REG). In all tests, a confidence level of 95 % was considered.

Method validation

The results showed full cone spray with an induced spray angle of 14.9˚. Spray characteristics analyse showed that distance influenced sprayed circle diameter, at the distance 24.5 cm from the nozzle the diameter of sprayed area was 70 mm and adjusting spray height at 12.5 cm decreased it to 50 mm. Transfer efficiency was significantly higher at the distance 12.5 cm (43.50 %) compared at the distances 24.5 cm (20.59 %; t=-9.59, df=148.7, $p < 0.0001$). Decreasing the distance from nozzle by 1.0 cm increased transfer efficiency almost 7 %. The transfer efficiency depended more on the volume ($r = 0.91$) than on pressure ($r = 0.23$; Fig. S2). The volume of the treatment had significant effect on transfer efficiency ($F = 177.71$, df=2, $p < 0.001$). The change of pressure had not significant effect on transfer efficiency ($F = 1.635$, df=2, $p = 0.21$).

The PCR identified the presence of dsRNA-GFP in all plant cultivars, plant parts and treatments, which were used regardless of surfactant use or not (Fig. 2).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Triin Kallavus: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Riina Kaasik:** Conceptualization, Methodology, Writing – review & editing, Resources. **Tõnu Leemet:** Writing – review & editing, Resources, Visualization. **Kaarel Soots:** Writing – review & editing, Resources, Visualization. **Liina Soonvald:** Investigation, Writing – review & editing, Visualization. **Silva Sulg:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Resources. **Eve Veromann:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2024.102734.

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