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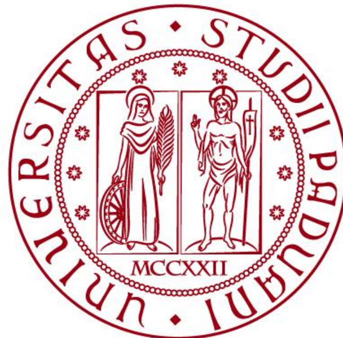

ELABORATO DI LAUREA

Application of a dsRNAs-induced RNAi method for acetolactate synthase (*ALS*) gene silencing in *Amaranthus hybridus* L.

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1. ABSTRACT

RNAi is a natural gene silencing pathway that can be taken advantage of for weed control in agriculture. Since RNAi can be triggered by the application of exogenous dsRNA to induce PTGS, the aim of the study is to develop a method for ALS (acetolactate synthase) gene silencing in Amaranthus hybridus L., a common weed that has become more resistant to herbicides over time, in order to inhibit its growth and avoid using herbicides. Thus, the technique can be considered as a sustainable alternative, fulfilling the EU new Green Deal requirements. In addition it is significant that by using exogenous dsRNA there's no need to resort to GM plants. It's also important to highlight the fact that the target is an endogenous gene and data shows that endogenous genes result less prone to silencing through exogenous dsRNA application. In this experiment dsRNA were produced using *in vitro* transcription and applied to plants' leaves using high-pressure spraying and mechanical inoculation. The only visible effect was a stain on a leaf that persisted 7 days post-treatment. Total RNA was extracted and its concentration and purity were verified using NanoDrop 2000c spectrophotometer (ThermoScientific).

2. STATE OF THE ART

2.1 RNAi mechanism in plants

RNAi is a conserved regulatory mechanism that enables sequence specific gene expression regulation (plant development, stress response), plant viral defense induction (RNAi slows down or terminates virus accumulation) and protection from insects and fungal infection through gene silencing. The silencing pathway can also be addressed to transposons and repetitive DNA by creating and maintaining heterochromatinic regions through epigenetic modifications. In the case of exogenous application, data suggests that dsRNAs are recognized as MAMPs and DAMPs and are perceived by cell-surface proteins called PRR taken up and transported into the cytoplasm via an unidentified mechanism. A RNase III enzyme (DICER-LIKE protein) cleaves dsRNAs into 20 to 25-nucleotide small interfering RNAs (siRNA) with 2-nt 3´ overhangs at both ends. Secondly, the antisense strand of the siRNA molecule is incorporated into an ARGONAUTE protein (AGO) thus forming the RNA-induced silencing complex (RISC). The siRNA molecule acts like a guide for RISC to scan the cytoplasm for recognition of cognate mRNA through complementarity-based interaction. This results in gene silencing in a sequence-specific manner via cleavage, destabilization, or translation repression of homologous mRNAs (Fig. 1).

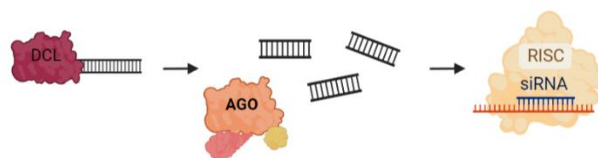


Figure 1

Next-generation sequencing data suggests that specific subsets of AGO proteins are related to each DCL and that also the stabilization of sRNAs into each AGO depends on the preferential binding affinity of each AGO for the 5′ terminal nucleotide of the sRNAs. Depending on the type of DCL protein, siRNAs can have different lengths and get involved in different pathways. If the dsRNA is cleaved by DCL4, the siRNA will be 21nt long and will associate with AGO1 to form the RISC complex, resulting in post-transcriptional gene silencing (PTGS). If the association occurs with DCL2, the dsRNA will be 22nt long and will associate with AGO1 and either recruit RDR6 – that enables the amplification of the silencing signal through generation of secondary siRNAs in the mechanism coined "transitive" silencing – or repress mRNA's translation. Finally, if the dsRNA is processed by DCL3, siRNAs will be 24nt long and loaded on AGO4. Cognate DNA recognition takes place, and Pol V is recruited to form ''RNA-directed DNA methylation'', but also histone modifications can occur (Das and Sherif, 2020).

A remarkable feature of siRNAs is the capacity to spread locally and systemically throughout the plant. A short-range movement through the symplastic route can spread 10-15 cell layers. This cell-to-cell spreading occurs most likely through plasmodesmata. Long-distance movement or systemic silencing has been found to be phloem-mediated and requires amplification of the silencing signals by RDRPs. The major RNAi-based crop improvement/protection strategies include the generation of dsRNA/hairpin RNA (hpRNA)-expressing transgenic plants and host-induced gene silencing (HIGS), which allows the silencing of genes in plant microbial pathogens or application of modified plant viruses inducing degradation of target plant mRNA, i.e., virus-induced gene silencing or VIGS. RNAi-based insect management strategies include generation of transgenic plants expressing dsRNAs targeting essential insect genes, feeding insects with dsRNAs, or plant foliar treatments with dsRNAs. However, the consequences of plant genetic modifications are not clear, which raises serious public concerns on their impacts on human health and environment and it is a costly and a complicated process for many horticultural crops. There also are legislative limitations on the cultivation of transgenic plants in many countries, including Italy. Concerning the VIGS, although it doesn't require genetic modifications of plants, this method presents limitations that prevent its wide application.

The research of an alternative approach for plant gene regulation without genomic modifications led to the investigation of exogenously induced RNAi, which has recently emerged as a promising method for regulation of essential genes in plant pathogens and for plant disease protection. For example, it is now well established that spraying plants with dsRNA or siRNAs encoding key genes of plant pathogenic fungi and viruses effectively reduces development of the pathogens and suppresses the infection process. Much less is known about the influence of exogenous dsRNAs/siRNAs on gene silencing in the plant genome. Several studies reported that external application of dsRNAs to Arabidopsis thaliana and siRNAs to *A. thaliana* or *Nicotiana benthamiana* triggered silencing of common plant transgenes, such as green fluorescent protein (GFP), β-

glucuronidase (GUS), yellow fluorescent protein (YFP), or neomycin phosphotransferase II (NPTII). Plant transgenes are known to be more prone for RNAi-mediated suppression in comparison with plant endogenes, and, therefore, targeting transgenes might be more achievable. Plant transgenes represent a good model for the studies aimed to affect the expression of plant genes due to higher transgene sensitivity for silencing, clearer transgene silencing effects, and a lower likelihood of secondary effect in comparison with the silencing of plant endogenous genes. To date there are just five investigations and a patent that showed that external plant treatments with naked dsRNAs led to downregulation of plant endogenous genes, including silencing of the 3-phosphate synthase (EPSPS) gene in tobacco and amaranth leaves, MYB1 gene in the orchid flower buds, Mob1A, WRKY23, and Actin genes in *Arabidopsis* and rice, two sugar transporter genes STP1 and STP2 in tomato seedlings, and a downy mildew susceptibility gene LBDIf7 in grapevine, CHS gene in *Arabidopsis*. According to the data, external plant dsRNA treatments led to the dsRNA uptake, reduced mRNA levels of the gene targets, and some phenotypic or biochemical changes. In addition, two studies reported using nanoparticles or laser light to ensure perception of exogenous dsRNA and downregulation of a plant endogenous gene by external dsRNA application. (Kiselev et al., 2021)

2.2 Aim of the study

The aim of the study is to develop a method for efficient stimulation of plant RNAi using exogenous dsRNA to silence ALS, an endogenous gene of *A. hybridus*. The objective is to achieve death of plants with an alternative method to ALS inhibitor herbicides, of which *A. hybridus* has developed resistance. The silencing of ALS gene should result in the inhibition of branched-chain amino acids biosynthesis and thus be lethal for amaranth plants. *A. hybridus L.* is, along with other weed species, prone to evolve resistance after repeated exposure to herbicides having the same site of action (SoA). The first European ALS-resistant amaranth was collected in soy-bean fields in north-eastern Italy in 2006. Since then, the area cultivated with soybean in Italy has substantially increased, resulting in a rising number of ALS-resistant Amaranthus cases. The main resistance mechanism appeared to be target-site mediated. The most common point mutations that cause *A. hybridus* to be resistant to herbicides are Trp_{574}Leu and Trp_{574}Met (Milani et al., 2020). Currently there is compelling evidence that plant transgenes are more sensitive to transcriptional and post-transcriptional silencing in comparison to endogenous genes for the following reasons: absence of introns and 5’ and 3’ untranslated regions (UTRs), which are known to suppress RNA silencing; higher expression (because transgenes are usually controlled by strong promoters) and thus higher production probability of aberrant mRNA, which are detected by plant RNAi machinery and further transcribed into secondary dsRNAs by RNA-directed RNA polymerase 6 (RDR6), resulting in transgene silencing amplification. In order to maximize the probability to obtain effective silencing, the chosen target gene is an

endogenous gene that shows comparable features to transgenes: ALS is indeed an intronless, nucleotide-stable, and single-copy gene. Three different fragments (E, B, F) of the Amaranthus hybridus acetolactate synthase (ALS) gene were amplified by PCR for in vitro transcription and dsRNA production. Each fragment localization was chosen to span the complete gene length in order to see if there was any variation in silencing efficacy based on the gene region targeted and the length of the fragment itself.

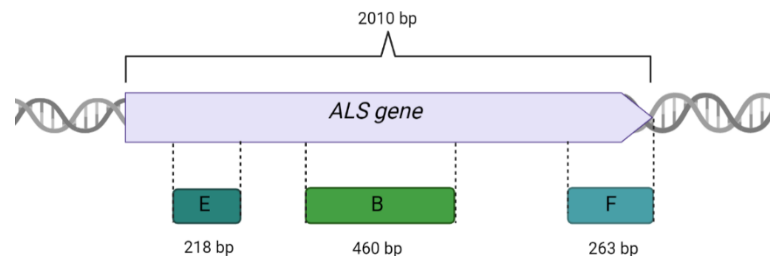


Figure 2

2.3 Compatibility of RNAi-based technology and sustainability

Herbicide resistant weeds are a major concern in modern agriculture, and this is exacerbated by the massive use of few active ingredients as herbicides over the years. Weed management systems based solely on herbicides are not sustainable in the long term, instead, programs that include different approaches are highly recommended, as stated by the EU commission (Directive 335 2009/128/EC and Regulation (EC) No 1107/2009 of the European Parliament and of the Council). In this context, the development of an efficient RNAi-based method would be suitable as a ''reactive'' strategy (restore herbicide susceptibility after resistance emergence) or even as a substitute for chemical herbicides, thus representing a sustainable alternative. Since the latter half of the last century, concerns have grown about the use of chemical herbicides, in particular their adverse effects on human and environmental health. Nowadays we are facing a rapid increase in human population – expected to reach 9 billion by 2050 – and adverse effects generated by climate change: growing demand for food, decrease in arable land, extreme weather events and widespread development of pesticide resistance are some of the reasons why a shift in agriculture towards sustainability should be urgently implemented (Fletcher et al. 2020).

The potential benefits of dsRNA application to induce RNAi are: lower toxicity than chemicals, species-specificity and a nominal environment impact with appropriate dsRNA design (eg. delivery mechanisms). DsRNAs are indeed unlikely to have undesired non-target effects, due to their high specificity and rapidity of degradation in the environment. Human uptake of dsRNA designed for a weed target is very unlikely to happen considering the multiple barriers in the human body. The Farm-to-Fork strategy is one of the pillars underneath the European Commission's new Green Deal and aims to ensure a more sustainable food production (Taning et al., 2021). One of the steps to achieve the aim is a reduction

in agrochemical inputs and exogenously applied dsRNA could definitely play a role in this.

3. EXPERIMENTAL APPROACH

3.1 Plant material and growth conditions

Seeds of Amaranthus hybridus L. were sown in Petri dishes containing agar (0.6%) and placed in a germination cabinet at 15/25°C night/day and 12 h photoperiod with neon tubes providing a photosynthetic photon flux density of 15-30 µmol m^{-2} s^{-1} (Scarabel et al., 2007). Pre-germinated seeds were transplanted into plastic pots (11 cm x 10.1 cm) filled with a standard substrate (60% silty loam soil, 15% sand, 15% perlite, and 10% peat by volume) and were grown in a greenhouse (30/20°C day/night) with a 16/8 h light/day photoperiod in Legnaro, north-eastern Italy (45°21'N, 11°58'E). All the plants involved in the experiment were at growth stage 14-16 of the extended BBCH Scale, corresponding to four- to six-leaf developmental stages at the moment of treatment.

3.2 Treatment

Given that most of the reports highlighted that dsRNAs within a size from 150- to 500-bp are the most efficient in inducing the RNAi pathway (Das and Sherif, 2020), it was decided to amplify: a 218-bp region at the 5'-end (named E), a 460-bp region at the centre (named B), and a 263-bp region at the 3'-end (named F). Each fragment localization was chosen to span the complete gene length in order to see if there was any variation in silencing efficacy based on the gene region targeted and the length of the fragment itself. A mixture of three dsRNA (E, B, F) diluted in a wetting agent (Silwet™ L-77 0.01%), H_2O, an osmolite compound (sucrose 200mM) and a buffer (MES 4mM) was applied on the abaxial surface of leaves in individual *A. hybridus* plants at a 4-6 leaves developmental stage. The procedure took place between 8:00 pm and 9:00 pm because, according to Kiselev et al. (2021), late time of day is optimal for the achievement of efficient gene silencing. Two application methods were performed: (1) mechanical inoculation by gentle rubbing the inoculation mixture with a pipette all over the leaf surface; (2) high pressure spraying using Conrad AFC-101°C 35mm atomizer at a distance of 2-3 cm from the leaf and at 8 bar pressure. When dsRNAs are delivered by high-pressure spraying, like in our experiment, the procedure is termed spray-induced gene silencing (SIGS). Mechanical inoculation was tested on a set of 8 plants using a different dosage for each subset of 4 plants. The first set received each dsRNA (E, B, F) in concentration of 83ng/µL corresponding to 10µg of dsRNA in 40µL per leaf (3 treated leaves per plant), while the second set received a higher concentration of dsRNA (293ng/µL of each dsRNA), corresponding to 35µg in 40µL. High pressure spraying was tested on a set of 4 plants using a dosage of 24 µL dsRNA in 2mL. In addition, a treatment with solely the wetting solution (Bayer formulation

containing H_2O, sucrose 200mM, Silwet™ L-77 0.01%, MES 4mM) was applied to a set of 8 plants to evaluate an eventual toxicity and the drying time of the wettable agents. In the first subset of 4 plants the application method was mechanical inoculation, and in the second it was high pressure spraying. A set of 4 plants remained untreated to represent the negative control, and a total of 24 *A. hybridus* plants were treated (Fig. 3).

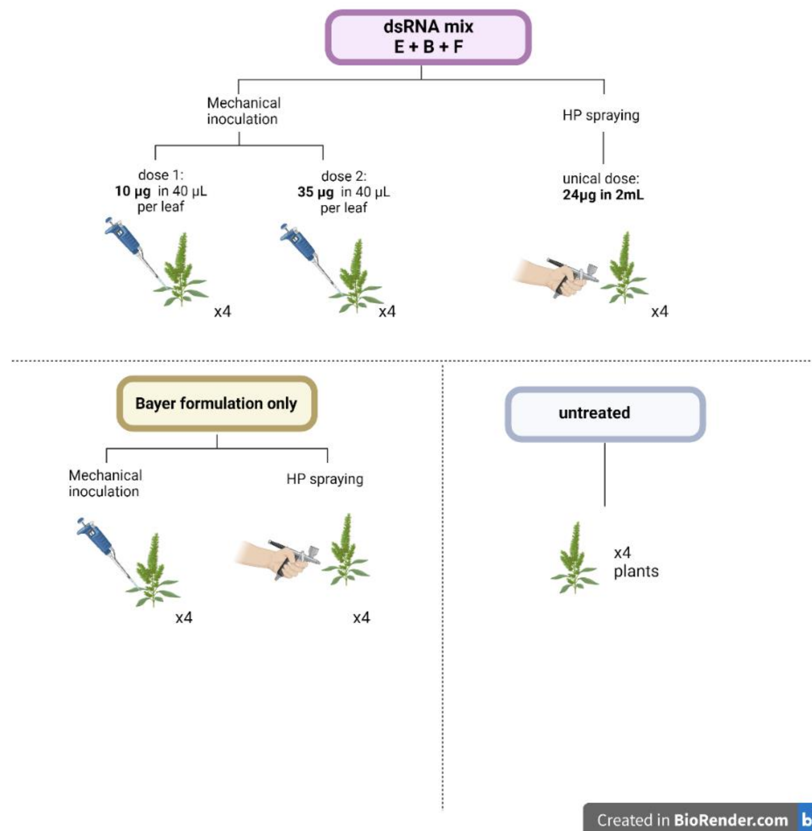


Figure 3

3.3 Sample collection

Samples of treated *A. hybridus* plants' leaves were collected at different time points, corresponding to: 24 hours post-treatment (T1), 72 hours post treatment (T2) and 7 days post treatment (T3). Samples were collected also before-treatment (T0) in order to further investigate the differences between the pre-treatment and post-treatment plant conditions. Immediately after excision, leaf samples were frozen in liquid nitrogen and stored at - 80°C in the laboratory freezer. The maintenance of low temperature is crucial for RNA conservation, in particular for the RNA molecules stability and for the inhibition of RNases. Before proceeding with the RNA extraction, leaves' samples had to undergo grinding. Two different methods were tested: pestle and mortar or drill. Both of them were performed in a refrigerated environment, using liquid nitrogen

to ensure low temperatures during grinding. The drill method enabled the attainment of a thinner powder sample in a smaller amount of time if confronted with the pestle and mortar technique.

3.4 Total RNA extraction

For extraction, the quantity of maximum 100mg of each sample was used. Total RNA extraction was carried out using two different kits: ''PureLink RNA Mini kit'' (Invitrogen), and ''RNeasy Plant Mini kit'' (Qiagen). Before using the Qiagen kit, the Invitrogen kit was also tested with different extracting conditions from the manufacturer's protocol. In the first of the 2 trials was omitted (table 1), while in the third was used β-mercaptoethanol coupled with a bigger quantity of lysis buffer (0,7 ml instead of 0,5 ml). The following subset of samples was submitted to RNA extraction in different conditions: samples from T1_1 to T1_8 were processed with the PureLink RNA Mini Kit (Invitrogen) following the manufacturer's directions; samples from T1_9 to T1_11 were incubated with the lysis buffer for 10 minutes, using the vortex every 3 minutes (table 4). The last subset of samples (from T_12 to T_14) were processed using Rneasy Plant Mini Kit (Qiagen) incubating with the RLT buffer for 3 minutes at 56°C (table 5).

After RNA purification samples were subjected to the optional step of on-column DNase I treatment, using Takara DNase I for 30 minutes at 37°C for the RNA extracted using the Invitrogen kit, while the RNA extracted with the Qiagen kit was treated with Qiagen DNase I for 15 minutes at 25°C.

Total RNA concentration and purity were verified using NanoDrop 2000c spectrophotometer (ThermoScientific) by measuring $OD_{260/230}$ and $OD_{260/280}$ ratio and making sure the values were respectively >1,8 and 1,8 – 2. Total RNA quality was evaluated via 1% agarose gel electrophoresis. Notably, the Qiagen RNeasy® Plant Mini Kit displayed a better performance in terms of higher RNA yield and quality (in terms of OD 260/230).

In respect to the gel electrophoresis, 18S and 28S ribosomal RNA (rRNA) bands were efficiently detected, in fact every run displayed two evident bands at 1000 – 1500 bp, as indicated by the 1kb Plus DNA Ladder. Less evident bands at lower molecular weight correspond to organellar RNA and rRNA 5S. The effective detection of distinct bands is an indicator of undegraded RNA, while the absence of detectable bands and the presence of a long smear would be a sign of RNA degradation. In correspondance of low molecular weight bands of the ladder there should be small RNAs. In detail, the dsRNAs that were used for treatment were 218, 263 and 460 bp long, so their band should appear below the 500bp molecular weight marker. DNA contamination can be observed in the T0_24 gel run, since there is a mild band at 10 000 – 15 0000 bp.

Since gel electrophoresis is not an efficient method to discriminate sRNA bands, the usage of other methods, such as Agilent 2100 Bioanalyzer, could have

improved the ability to detect small RNAs (in particular dsRNAs, which are central in this study) and to analyze the quality of samples at the same time.

Sample	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
T0_1	231,5	2,17	1,4
T0_2	96,0	**2,15**	**2,1**
T0_3	214,0	2,17	1,6
T0_4	11,1	2,15	1,13
T0_5	57,0	2,22	0,14
T0_6	181,4	**2,15**	**2,18**
T0_7	100,5	2,15	1,65
T0_8	217,2	2,18	0,71

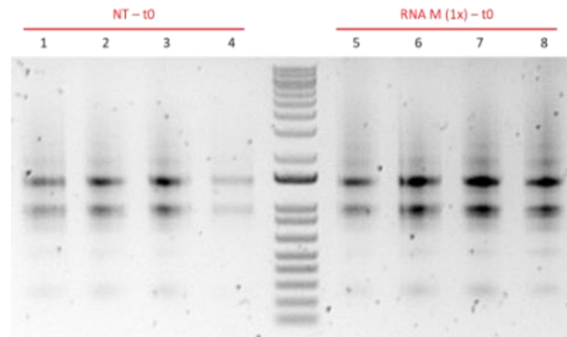


Table 1: (11/04/2022) only two of the samples showed acceptable values of $OD_{260/230}$ and $OD_{260/280}$

Sample	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
T0_9	239,9	2,15	1,08
T0_10	47,8	2,18	0,42
T0_11	47,0	2,15	0,46
T0_12	144,6	2,16	1,03
T0_13	40,1	**2,17**	**1,80**
T0_14	95,0	2,16	0,88
T0_15	270,7	**2,15**	**2,40**
T0_16	184,2	2,15	1,37

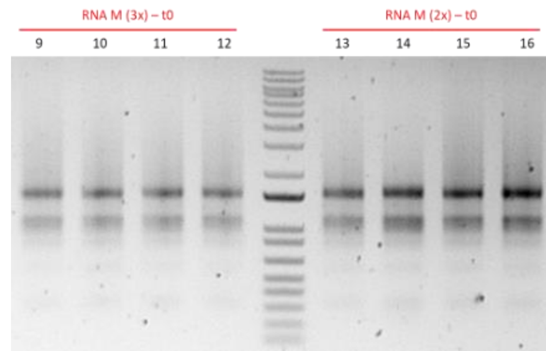


Table 2: (13/04/2022) only two of the samples showed acceptable values of $OD_{260/230}$ and $OD_{260/280}$

Sample	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
T0_17	232,7	**2,15**	**2,07**
T0_18	266,1	**2,15**	**2,24**
T0_19	59,4	**2,15**	**1,84**
T0_20	18,9	2,12	0,65
T0_21	65,0	2,14	1,03
T0_22	334,6	**2,14**	**2,23**
T0_23	139,8	**2,14**	**2,12**
T0_24	60,0	2,16	1,25

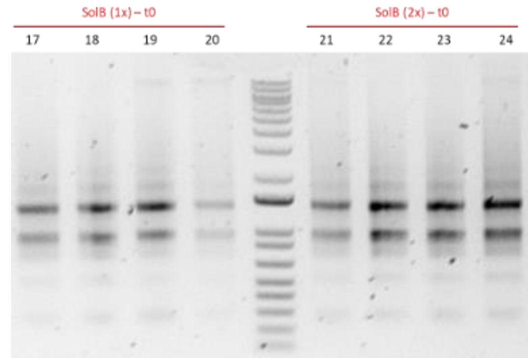


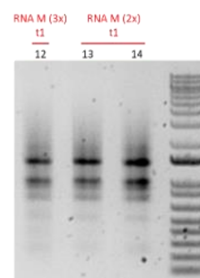
Table 3: 5 of the samples showed acceptable $OD_{260/230}$ and $OD_{260/280}$ values

Sample	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
T1_1	3,8	2,76	0,33
T1_2	9,7	2,24	0,46
T1_3	37,8	**2,18**	**2,03**
T1_4	5,4	2,04	0,55
T1_5	7,9	2,21	1,05
T1_6	138,5	**2,13**	**2,39**
T1_7	60,3	**2,16**	**1,84**
T1_8	11,1	2,23	0,56
T1_9	29,4	**2,37**	**2,32**
T1_10	52,9	2,21	2,07
T1_11	147,8	2,18	1,63

Table 4: low quality and concentration of RNA led to the decision of not running the gel

Sample	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
T1_12	131,4	**2,16**	**1,98**
T1_13	86,5	**2,16**	**1,9**
T1_14	171,1	**2,16**	**2,31**

Table 5



4. RESULTS AND DISCUSSION

4.1 In vitro dsRNA transcription

In their review, Das and Sherif, (Das and Sherif, 2020) highlighted that dsRNAs within a size from 150- to 500-bp are the most effective in inducing the activation of the RNAi pathway. For this reason it was decided to produce by *in vitro* transcription three different dsRNAs of various lengths, ranging from 218- to 460-bp, targeting three distinct ALS (GenBank MH036306.1) regions: the 5'- and 3'-ends, and a central region. In particular, the fragments were named E, B and F, and measured 218-, 460- and 263bp respectively.

Before proceeding with *in vitro* transcription, DNA templates concentration and purity were determined by measuring OD260 and OD260/280 ratio, respectively, on a NanoDrop 2000c spectrophotometer (Thermo Scientific) NanoDrop 2000c. DsRNA molecules were produced by cloning the three different fragments of ALS gene sequence in a L4440 vector and thus using the HighYield T7 RNAi kit (Jena Biosciences) for *in vitro* transcription. Finally, dsRNA were excised from vectors using restriction fragments (HindIII and NotI) and subsequently analyzed by 1,2% agarose gel electrophoresis.

4.2 Phenotypical evaluation of treated plants

After treatment, amaranthus plants were observed daily in order to notice any phenotypical alteration. Since ALS gene is crucial for the synthesis of branched-chain aminoacids, some signs of decay and ultimately death was expected to be observed in plants treated with the dsRNAs mixture. However, alterations were not displayed by either treated and untreated plants, exception made by one plant that was treated with the dsRNA mixture by high-pressure spraying. One of its leaves showed an irregular stain on the abaxial surface (Fig. 3) that persisted even 7 days post treatment, probably ascribing to the mark of high-pressure spraying and not just to a wetting effect of the solution. The RT-qPCR analysis of this leaf could be influenced by the presence of expressed genes related to the wound repair mechanism. Any other differences between plants treated with different doses and methods were not visible.

4.3 Considerations about factors influencing dsRNA efficacy

In this paragraph is presented a discussion about the possible causes of the unsuccessful outcome of the experiment. Appropriate plant age, late time of day, low soil moisture at the moment of dsRNA application, and optimal dsRNA application modes are important for efficient gene silencing induced by direct foliar dsRNA treatments (Kiselev, 2021): in this experiment these parameters have been respected. In general, exogenous application methods include infiltration, injection, spreading, mechanical inoculation, and root/seed soaking and high pressure spraying, which is equally efficient but more convenient and user-friendly method for dsRNA delivery than particle bombardment: high-pressure spraying by using a conventional compressor and an airbrush pistol prevents from extensive shearing, which could be a drawback associated with biolistic. (Dalakouras et al., 2016). High-pressure spraying is also more efficient than wiping and infiltration, however, the efficacy can be increased by using nanotechnology-based delivery and surfactants-based delivery methods. The delivery of exogenous dsRNAs into plant cells is considered the most crucial step in initiating RNAi machinery; indeed complex cellular structures such as the rigid cell wall act as a physical barrier to provide tensile strength and protection against several stresses.

Even though it has been demonstrated that siRNAs can be successfully introduced in the plant by high-pressure spraying and induce silencing of a transgene (Dalakouras 2016), endogenous genes appear less susceptible to transcriptional and post-transcriptional silencing. The reason is that plants have developed reliable mechanisms to protect endogenous genes thus ensuring their survival. There is a limited number of investigations reporting on the silencing of plant endogenous genes after exogenous dsRNA or siRNA application. At present there are five investigations and a patent that showed downregulation of plant endogenous genes upon external plant treatments with naked dsRNAs. The first report wherein exogenous RNA application into plants triggered RNAi of a plant gene was described in a 2011 Monsanto patent. The studies that reported endogenous gene silencing regarded: *MYB1* gene in the orchid flower buds, *Mob1A*, *WRKY23* and *Actin* genes in *Arabidopsis* and rice, two sugar transporter genes *STP1* and *STP2* in tomato seedlings, a downy mildew susceptibility gene *LBDIf7*, and a glutathione S-transferase *GST40* gene in grapevine.

In another experiment (Dubrovina et al., 2019) the effects of different dsRNA concentrations (0.1, 0.35 and 1.0 µg/µl) were analyzed and the results indicated that optimum concentration (0.35 µg/µl) had a higher significant influence on transgene-silencing efficiency.

The factors affecting exo dsRNAs application efficacy concern the stability of the molecule before and after plant uptake, the uptake itself and the transportation throughout the plant. Indeed, to reach the RNAi machinery, dsRNA must traverse the plant cuticle, avoid nuclease degradation and penetrate the cell wall and plasma membrane (Bennet et al. 2020). The plant cuticle has a lipophilic nature and has the specific purpose of protecting plants leaves from water and solutes

entrance. This is the reason why in the experiment dsRNA were diluted in a solution containing a wetting agent, Silwet L-77 0.01%, which is a non-ionic surfactant that has the ability to reduce surface tension. In some experiments (Bennet et al., 2020), endogenous gene silencing was obtained by including ≥0.3% of a surfactant such as Silwet L-771 (this concentration is not employed in commercial agriculture): it is noticeable that the concentration used in our experiment is lower than the concentration used in other successful experiments. Additionally, it is known that dsRNA must penetrate the cell wall in order to reach the symplast and spread through plasmodesmata to adjacent and distant cells, and the wetting solution may be useful to overcome the barrier of the cuticle, but might not be enough for the other barriers, such as the cell wall.

The capacity to trigger an effective RNAi response also depends on the following dsRNA molecule features: length, presence of overhangs, potential formation of secondary structure in plant mRNA that could limit accessibility to dsRNA. The effects of different lengths of dsRNAs (315, 596, and 977-bp) targeting different virus genes were also investigated in *N. tabacum* leaves and results indicated that shorter dsRNAs showed reduced antiviral activity, indicating that dsRNA length could influence on its efficacy. Effective RNAi relies on the signal-amplifying action of a specific RNA-dependent polymerase (RdRP) capable of converting exogenously encountered dsRNAs into an abundant internal pool of secondary siRNAs.

The major limitation of exogenous applications of naked-dsRNAs is their short-term stability, which in the agronomic formulation is a point of major concern because the molecule must join the RISC complex in the plant and induce the silencing machinery. Some studies reported that nanoparticle-based delivery could enhance the stability and efficacy of exogenously applied dsRNAs when compared to naked-dsRNA delivery. The stability of exogenously applied naked-dsRNAs was also compared with layered double hydroxide (LDH) clay nanosheets-based delivery. Confocal microscopic analyses of Cy3 fluorophore labeled naked-dsRNA and LDH-loaded dsRNA targeting CMV2b found that most of the naked-dsRNA was washed away, whereas LDH-loaded dsRNA largely remained on theleaves. This study also reported that LDH-loaded dsRNA showed sustained release and were detectable after 30 days of application on sprayed *N. tabacum* leaves, but the naked dsRNA was nearly undetectable after 20 days. Therefore, it was suggested that LDH nanosheets-based delivery can significantly improve the stability of exogenous dsRNA. Based on the above-mentioned studies, nanoparticle based delivery techniques could facilitate the delivery of exogenous dsRNAs by increasing their stability and uptake (Das and Sherif, 2020). However, it is worth noting that nanotechnology is quite an expensive technology and also sensitive to the encapsulation process.

In the current literature, there is a lack of data on the plant uptake and processing of exogenously applied dsRNAs. Although the mechanisms underlying the recognition and cellular uptake of extracellular DNA by receptors have not been identified in plants, a study showed that purified dsRNAs from virus-infected

plants and synthetic dsRNA analogs, both induced PTI responses in Arabidopsis. Exogenous dsRNA-induced PTI responses were dependent on the co-receptor SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1) but were DCL-independent. Thus, it was proposed that membrane-bound SERK1 could act as a potential dsRNA receptor. In this regard, global transcriptomics and proteomics analysis may help to screen trans-membrane marker proteins and genes for elucidating the receptor-mediated recognition, perception, and uptake of dsRNAs into plant cells.

4.4 To do next

The next passages to carry out for a better understanding of the mechanism underlying uptake, processing and efficacy of exogenously applied dsRNA for ALS silencing in *A. hybridus* will be described in this paragraph. Firstly, a retrotranscription and RT-qPCR are required to evaluate the expression of the ALS and RNAi pathway genes and eventually notice a down-regulation and an up-regulation, respectively. It should be noted that in the case of the leaf that shows a brownish stain, the gene expression profile could be altered due to the activation of the wound-repair mechanism. These two analyses are scheduled for the next months. In addition, RNA-Seq analyses would be desirable to evaluate potential enrichment of foliar sRNA after exogenous dsRNAs application and for the investigation of quantity and quality of transcripts potentially associated with uptake and processing. Indeed, there are quite limited studies where RNA-seq or other approaches were applied to analyze the processing of exogenous dsRNAs applied to plant surfaces, while such studies are required to uncover the molecular mechanisms of exogenously induced RNAi. For example the first study where RNA-Seq was used in plants to evaluate exo dsRNAs efficacy for gene silencing dates back to may 2022 (Nityagovsky et al., 2022), and it showed that foliar application of both AtCHS- and NPTII-dsRNAs led to the emergence of a large number of sRNAs mapping to the AtCHS and NPTII, which was not detected after the control water treatments. Finally, the execution of a degradome sequencing would provide a comprehensive means of analyzing patterns of RNA degradation, in particular the cleavage site in the target, and provides additional information for the study of RNA processing, such as information regarding RNA-binding proteins.

4.5 Future perspectives: factors to take in consideration before a potential commercialization of dsRNA based biopesticides

Limitations for a commercially viable product to overcome include stable delivery of the topically applied dsRNA and extension of the effect duration besides reasonable costs. The production of dsRNAs is based on in vitro (commercial kits) or in vivo methods that require the DNA dependent RNA polymerase (DdRP) from bacteriophage T7 for transcription of target-specific sequences. So far, the commercial kits used for dsRNAs production are quite expensive, limited to small-scale production, and

prone to false amplification, which may lead to poor quality of dsRNA products. The production of dsRNAs using in vivo methods involves the use of bacteria (e.g. Escherichia coli and Pseudomonas syringae) and yeast (*Yarrowia lipolytica*). "RNAgri" agricultural industry developed microbial fermentation technology to manufacture dsRNAs at a larger-scale. This industry utilizes a protein to bind the desired RNAs, hence protecting them against degradation. The final dsRNA products are considered safe to use and more stable than naked dsRNAs. In comparison to the *in vitro* transcription system, microbial-based dsRNAs production by prokaryotic or eukaryotic cells is considered as a sustainable strategy for providing large quantities of dsRNAs. More recently, to meet the high market demand, several industrial companies are now shifting to microbial-based production systems to manufacture dsRNAs at a large-scale and nearly at 2 USD/gm. Using bacterial minicells is another promising technology that is currently utilized for the production and encapsulation of dsRNAs. If successful, this technology could provide better shielding and slow and sustained release of dsRNAs for agricultural purposes under open-field conditions (Das and Sherif, 2020).

Development of RNA-based biopesticides is gaining momentum as a narrow-spectrum alternative to chemical-based control measures, with infestants and pathogens targeted with accuracy and specificity. Risk identification is another key step to include in potential dsRNA biopesticide development. RNAi-based products have higher selectivity and better safety profiles (less mobile through the soil, less persistent, less toxic) compared with contentious chemical products. Although selecting effective RNAi targets can be a challenging step, a combination of *in silico* tools and an increase in the availability of genome databases for various species has made it possible to design species-selective and efficient dsRNA molecules with zero to negligible off-target effects in non-target species. Additionally, RNA is a molecule that is rapidly degraded by nucleases and UV radiation. Anyway, the biosafety evaluation should be carried out on a case-by-case basis. Another important point is to take into account the public perception of RNAi based products. Perceived benefits alone might not be the decisive factor in societal acceptance, for this reason it is suggested to include the perception of both risks and benefits to capture also trade-offs that consumers are willing to make to accept new generations of herbicides and pesticides. Findings from such evaluations would further shape communication strategies that could drive societal acceptance.

4.6 Conclusions

The increasing human population and discussions about the safety of transgenic plants promote the development of new strategies to regulate plant properties without genomic manipulations. Plant foliar treatment with dsRNA has considerable potential as an innovative approach for gene regulation in plants and for plant pathogen control to ensure low-risk and environmentally friendly plant management.

It should be noted that most of the studies on the efficacy of exogenously applied dsRNAs were carried out under set experimental conditions, e.g., using detached leaves, targeting of transgenes, co-inoculation of dsRNAs with target viruses, and have rarely been implemented under open field conditions where several factors can largely affect their stability, uptake, and overall applicability.

Further investigations are required for elucidating the uptake mechanism at a molecular level, because there could be missing information that could be useful to design an effective application and delivery technology. Secondly, more experiments of naked dsRNA foliar application by high-pressure spraying are needed to validate the fact that this method is actually effective on *A. hybridus* plants.

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