agronomy

MDPI

Article

Enhanced Herbicidal Action of Clopyralid in the Form of a Supramolecular Complex with a Gemini Surfactant

Alla B. Mirgorodskaya [1,*], Rushana A. Kushnazarova [1], Lucia Ya. Zakharova [1], Alana A. Ulyanova [2], Dmitry Y. Litvinov [2,*], Andrey O. Blinkov [2], Mikhail G. Divashuk [2], Irina A. Kochanova [3] and Liliya M. Nesterova [3]

[1] Arbuzov Institute of Organic and Physical Chemistry, FRC Kazan Scientific Center of RAS, Arbuzov Str. 8, Kazan 420088, Russia
[2] All-Russia Research Institute of Agricultural Biotechnology, Timiryazevskaya Str. 42, Moscow 127550, Russia
[3] Joint Stock Company "AUGUST" Inc., 6, Tsandera Str., Moscow 129515, Russia
* Correspondence: mirgoralla@mail.ru (A.B.M.); dlitvinov@gmail.com (D.Y.L.)

Abstract: Surfactants are often added to herbicidal formulations to improve the delivery of the herbicide into plants. In this study a new herbicidal formulation was formed based on the clopyralid with 0.01% gemini surfactant hexanediyl-1,6-bis(dimethylcetylammonium bromide) (16-6-16) as an adjuvant. The increase in the efficiency of the formulation was associated with the formation of a supramolecular surfactant–herbicide complex (SMC), which has improved wetting properties, provides high clopyralid concentration on the leaf surface, and has higher penetrating ability compared to surfactant-free clopyralid solutions. Comparison of the herbicidal action of clopyralid–16-6-16 SMC with two commercial formulations of the same concentration of clopyralid was performed using digital phenotyping of the model weed plant cocklebur (*Xanthium strumarium*). Based on the spectral indices NDVI (normalized differential vegetation index) and PSRI (plant senescence reflectance index) and key morphological indexes of the leaf angle, plant height, and leaf area, we showed that clopyralid formulations strongly affected the plants and that the strongest and most durable effect was exerted by the clopyralid–16-6-16 SMC formulation.

Keywords: herbicides; adjuvant; dicationic surfactant; wetting; digital phenotyping

check for updates

Citation: Mirgorodskaya, A.B.; Kushnazarova, R.A.; Zakharova, L.Y.; Ulyanova, A.A.; Litvinov, D.Y.; Blinkov, A.O.; Divashuk, M.G.; Kochanova, I.A.; Nesterova, L.M. Enhanced Herbicidal Action of Clopyralid in the Form of a Supramolecular Complex with a Gemini Surfactant. *Agronomy* **2023**, *13*, 973. https://doi.org/10.3390/agronomy13040973

Academic Editor: Leslie A. Weston

Received: 24 December 2022
Revised: 2 March 2023
Accepted: 21 March 2023
Published: 25 March 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

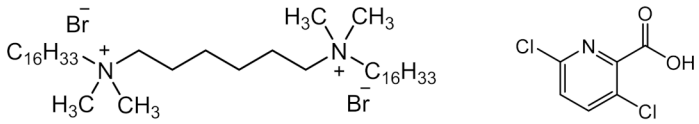
1. Introduction

Herbicides are widely used to increase crop yields, but they have a negative ecological impact. The most direct way to reduce the herbicidal load on the environment is to increase the effectiveness of their action by improving the delivery of herbicides into plants. One of the promising strategies is to use the surfactant–herbicide formulations for this purpose [1]. Surfactants in herbicide formulations act as adjuvants, improving the wetting of plant surfaces, providing better solubility of the active ingredients in water and increasing their local concentration, thereby reducing the total loading of chemicals, as well as facilitating their transport into the plant [2–4]. In most cases, non-ionic surfactants are used, which are non-toxic, good dispersing agents, stable under normal conditions, and can be easily mixed with any pesticide [5–9]. Cationic surfactants have a number of advantages over non-ionic surfactants, such as higher surface activity, greater solubilization capacity, and better ability to bind efficiently to biosurfaces, making them attractive for agricultural use [10–12]. In addition, cationic surfactants exhibit significant antimicrobial activity, including the ability to fight plant diseases caused by fungi and bacteria [13–15]. Nevertheless, they are used less often than non-ionic surfactants due to their higher toxicity, which may carry certain environmental risks [16].

In the search for a compromise between efficacy and environmental risks when using cationic surfactants as adjuvants, one can turn to compounds with reduced toxicity or which show their functional activity within a low concentration range. In this regard,

dicationic gemini surfactants have significant potential. This class of compounds, which appeared at the turn of the 20th–21st centuries, currently attracts a lot of research attention due to a number of unusual properties that are based on their molecular structure. Gemini surfactants contain two head groups linked by spacers of various lengths and structures and two hydrophobic tails linked to the head groups [17–19]. Unlike their monomeric counterparts, they have an order of magnitude lower values of the critical micelle concentration (CMC) and are characterized by significant conformational mobility and the ability to form aggregates of various morphologies with high positive charge on their surface. A set of attractive properties of dicationic surfactants, such as an extremely low working concentration range, large solubilization capacity, and high antimicrobial activity [20–23] lie behind their wide practical applications.

Recently, some studies have reported the positive effect of gemini surfactants on the effectiveness of herbicides, clopyralid in particular [24,25]. Clopyralid is a systemic selective herbicide of the auxin mimic family that targets broadleaf weeds [26]. These herbicides selectively act on a number of dicotyledonous weeds, such as thistle (*Cirsium arvense*, *Cirsium vulgare*, *Silybum marianum*, *Carduus crispus*), chamomile (*Matricaria perforata*), dandelion (*Taraxacum officinale*) and clover (*Trifolium repens*, *Trifolium pratense*), but do not affect monocots and some cultivated dicots, such as cruciferous plants or strawberry leaves [27–29]. We have previously shown that the use of the gemini surfactant hexanediyl-1,6-bis(dimethylcetylammonium bromide) (16-6-16) promotes the transport of clopyralid into the plant [25] (Figure 1).



16-6-16 **Clopyralid**

Figure 1. Structural formulas of hexanediyl-1,6-bis(dimethylcetylammonium bromide) (16-6-16) and clopyralid.

The objectives of the present study were to (1) evaluate the efficacy of the supramolecular complex of the clopyralid and 16-6-16 gemini surfactant (herein referred to as "Clopyralid SMC") compared to commercially available clopyralid formulations, and (2) to evaluate whether digital phenotyping could reveal differences in performance between herbicide formulations in a short-term study.

We prepared and characterized the supramolecular complex of the clopyralid and 16-6-16 gemini surfactant and tested its herbicidal action on the model weed cocklebur (*Xanthium strumarium*), which, like many other weeds, belongs to *Asteraceae* family and infests wheat and other fields in the central part of European Russia.

In a pot experiment, the effectiveness of clopyralid SMC was compared with Hacker clopyralid formulations that are currently used in agriculture. Morphological and spectral changes of the plants were measured using the digital phenotyping approach. Measurements carried out using digital phenotyping do not affect the physiological state of the studied plants and provide accurate quantitative information about the spatial and spectral characteristics of plants. Using a 3D scanning multispectral digital phenotyping system, we collected time-series data to compare the effect of the Clopyralid SMC and two commercial formulations of clopyralid with equal concentrations of the clopyralid on plant height, leaf area, leaf angle, digital biomass, and spectral indices based on the reflectance of plants in the near infrared (NIR), red, green, and blue spectral regions, characterizing the state of chlorophyll and other plant pigments.

2. Materials and Methods

Clopyralid formulations Hacker WG (water-dispersible granules) and Hacker 300 SL (soluble liquid) were obtained by JSC August Inc. (Moscow, Russia) from clopyralid

(CAS 57754-85-5). The gemini surfactant 16-6-16 was synthesized by the reaction of hexadecyl bromide (CAS 112-82-3) with N,N′-tetramethylhexamethylenediamine (CAS 111-18-2) followed by recrystallization from acetonitrile, similar to the method of Mirgorodskaya et al. [30].

2.1. Analysis of Surface Properties and Aggregation Processes in Solutions of Surfactants

The surface tension of solutions 16-6-16 with and without clopyralid was determined by the Wilhelmy method (plate tear-off) using a K100 tensiometer (KRUSS, Hamburg, Germany).

The dynamic contact angle was determined on a K100 tensiometer by measuring the force expended when a solid sample (Parafilm M tape) is immersed in the test liquid with a known surface tension.

The spreading area of the surfactant solution over the surface of the Parafilm M tape was determined for drops of a fixed volume deposited on it (0.06 mL). For better visualization of the spreading drops, a water-soluble dye, rhodamine, was added to the tested solutions. The experiment was carried out at least five times to ensure the reliability of the data obtained.

Particle sizes in solutions were determined on a Malvern ZetaSizer Nano dynamic light-scattering photon-correlation spectrometer (Malvern Instruments, Malvern, UK). The light source was a 10 mW He–Ne gas laser with a wavelength of 633 nm. The light-scattering angle was 173°. The pulse accumulation time was five to eight minutes. Signals were analyzed using a single-plate multichannel correlator connected to a PC equipped with the software package version 7.11 for estimating the effective hydrodynamic radius of dispersed particles. All samples were analyzed in triplicate; the average error of measurements was approximately 4%.

All measurements described in this subsection were carried out at a temperature 25 ± 0.5 °C.

2.2. Plants and Treatments

The common cocklebur (*Xanthium strumarium*), a dicotyledonous weed common in wheat fields of the Lipetsk and Voronezh regions of the Russian Federation (51°40′18.0″ N, 39°12′38.0″ E and 52°36′11.0″ N and 39°34′15.0″ E, respectively), was used as a model plant in the study.

Plants for the experiment were grown in pots 16.5 cm long, 9.5 cm wide and 8.5 cm high, two plants per pot, in a universal soil containing all the necessary macro- and microelements. The soil was moistened with water in an amount of 50% of its mass, and the moisture was maintained by watering three times a week with randomly selected pots weighed once a week. The plants were grown indoors at +22°C and 60% relative humidity with 16 h of daylight.

The day of treatment was taken as day 0. On this day, all plants were treated by spraying water or aqueous clopyralid formulations (20.6 mL/m^2) in five replicated pots for each treatment: Control (water), Hacker WG, Hacker 300 SL, and Clopyralid SMC. All formulations contained the herbicide clopyralid (active ingredient) at a weight percent concentration of 0.03% (0.03% wt.) but differed in the type of formulation (composition) (Table 1). The choice of the surfactant concentration used in the Clopyralid SMC (0.01%) assumed the optimal ratio between its consumption and the need for the formation of a SMC of clopyralid, based on the data of Mirgorodskaya et al. [25].

Table 1. Clopyralid formulations used in the study.

Name	Composition
Hacker WG	Clopyralid potassium salt, 0.03% wt. by acid, from water-dispersible granules
Hacker 300 SL	Clopyralid monoethanolamine salt, 0.03% wt. by acid, from soluble liquid
Clopyralid SMC	Clopyralid acid (0.03% wt.), 16-6-16 (0.01% wt.), supramolecular complex

2.3. Digital Phenotyping

Morphological and spectral measurements were started immediately after the plants were treated with clopyralid formulations. The measurements were carried out using the TraitFinder digital phenotyping system equipped with two PlantEye 3D scanners (Phenospex, Netherlands). Each of the PlantEye scanners, when moving along the bench with plants, repeatedly passes a 940 nm laser beam in the direction perpendicular to the movement, captures the light scattered by the objects and, using triangulation, builds a 3D model of the objects on the bench. Two 3D plant models captured by PlantEye scanners from two angles are combined into one model that is more complete than the original ones. The resolution of the 3D model is higher than 1 mm in each of three axes. In addition to the laser, objects are scanned in a similar way using four LED light sources in the blue (472 nm), green (535 nm), red (632 nm), and near infrared (NIR, 735 nm) regions of the spectrum. Light scattered by objects from the LEDs is captured and added to the spatial data, which gives information about the reflection at different spectral regions for each point of the 3D model. Based on the 3D model, the HortControl software generates morphological data—leaf area, plant height, digital biomass, leaf angle, etc. Based on the reflection data in specific spectral regions, spectral indices are calculated:

$$NDVI = (R_{NIR} - R_{RED})/(R_{NIR} + R_{RED}) \quad (1)$$

where NDVI is the Normalized differential vegetation index, R_{NIR} indicates the reflection in the NIR (735 nm) region, and R_{RED} indicates the reflection in the red (632 nm) spectral region.

$$PSRI = (R_{RED} - R_{GREEN})/R_{NIR} \quad (2)$$

where PSRI is the Plant senescence reflectance index, and R_{RED}, R_{GREEN}, and R_{NIR} indicate reflections in the red (632 nm), green (535 nm), and NIR (735 nm) spectral regions, respectively.

TraitFinder determines the spectral indices for each individual point in the 3D plant models. Based on these data, the average values of the indices are calculated. In addition, the proportion of a plant with an index value in a given range, called a "bin", is determined. In this study, for all spectral indices, "bin 2" is the fraction of leaves with indices between minus 0.5 and 0, "bin 3" is the fraction of leaves with indices between 0 and 0.5, and "bin 4" is the fraction of leaves with indices between 0.5 and 1.

2.4. Data Representation and Statistical Analysis

Digital phenotyping data was plotted and statistically evaluated using the Jupyter Notebook platform. This utility grouped measurements close in time into clusters using the univariate k-nearest neighbor method on the full set of experimental time points and calculated the average value after removing outliers using the interquartile range three sigma test. All digital phenotyping data were analyzed and presented as changes from the initial values (first measurements on day 0). Statistical significance between treatments over the entire length of the study was calculated with a paired Student's test. Differences between individual measurements at specific time points were analyzed using ANOVA and the post-hoc Tukey HSD test with significancy criteria cut-off FWER = 0.05. The plots were drawn with smoothing by spline 8k 8d.

3. Results and Discussion

3.1. Characterization of the Solution of Clopyralid and Gemini Surfactant 16-6-16

3.1.1. Wetting and the Surface Properties of the Solution of Clopyralid and Gemini Surfactant 16-6-16

The positive effect of surfactants on the effectiveness of agrochemicals is usually associated with their ability to reduce surface tension at the phase boundary, which leads to an increase in wetting action and provides more efficient contact of the plant surface with the herbicide solution [31,32]. The influence of 16-6-16 on the wetting effect of clopyralid solutions was studied by using the Parafilm M tape. This film mimics the waxy cuticle of the leaves yet is uniform, allowing large area samples to be tested with statistically significant results. The spreading area (S) of a test solution droplet on the film surface was chosen as a measure to evaluate the wetting efficiency of the solution. The role of surfactants can be judged from the observed value of S (Table 2, Figure S1), which increases by more than three times when 16-6-16 is added to aqueous solutions at a concentration of 0.01%. This is in accordance with a decrease in the contact angle (θ) and surface tension (γ) (Table 2, Figure S1). It is noteworthy that the presence of clopyralid enhances the surface properties of 16-6-16, as evidenced by the lower values of contact angle and surface tension in solutions containing both reagents.

Table 2. Surface properties of clopyralid and hexanediyl-1,6-bis(dimethylcetylammonium bromide) (16-6-16) solutions: spreading area (S), contact angle (θ) and surface tension (γ).

Solution	S_i/S_0 [1]	θ, °	γ, mN m^{-1}
Water	1	101.3	72.2
Clopyralid 0.03% wt.	1	106.3	68.6
16-6-16 0.01% wt.	3.1	83	43.6
Clopyralid SMC	3.4	74	38.8

[1] Spreading area of a drop (S_i), normalized to water (S_0).

3.1.2. The Clopyralid/16-6-16 Supramolecular Complex

Since clopyralid is an acid and dissociates in aqueous solutions, its anionic form can interact with cationic surfactants in monomeric or aggregated forms due to electrostatic forces, forming a supramolecular complex. The interaction of clopyralid with 16-6-16 in solution is also observed in the aggregation properties of the solution of 16-6-16. Surface tension isotherms for the systems based on this surfactant are shown on Figure 2. It follows from these that the addition of clopyralid at a concentration of 0.03% to an aqueous solution of a gemini surfactant not only leads to a more significant decrease in surface tension, but also affects the value of CMC, increasing it almost two-fold (Table 3).

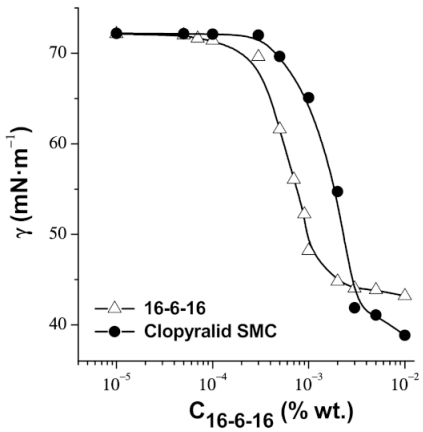


Figure 2. Surface tension isotherms of 16-6-16 aqueous solutions in the presence and absence of clopyralid at 25 °C.

Table 3. Properties of 16-6-16 aqueous solutions in the absence and presence of clopyralid: CMC, hydrodynamic diameter (D_h, nm) of particles, and polydispersity index (PdI).

Solution	CMC, %wt.	D_h, nm	PdI
16-6-16 0.01% wt.	0.0013	3.5	0.648
Clopyralid SMC	0.0029	7.2	0.325

The dynamic light-scattering data of micelles in a solution of 16-6-16 alone and together with clopyralid also indicate the interaction between the surfactant and clopyralid, leading to the formation of the complex. The addition of the herbicide to the 16-6-16 solution leads to an increase in the size of aggregates present in the solution and a decrease in polydispersity (Figure 3, Table 3). Overall, gemini 16-6-16 in aqueous solution forms bigger micelles with clopyralid, demonstrating a narrower size range and better surface properties than 16-6-16 alone. We designated here the formulation of clopyralid 0.03% wt. with 16-6-16 0.01% wt. as the clopyralid supramolecular complex (Clopyralid SMC).

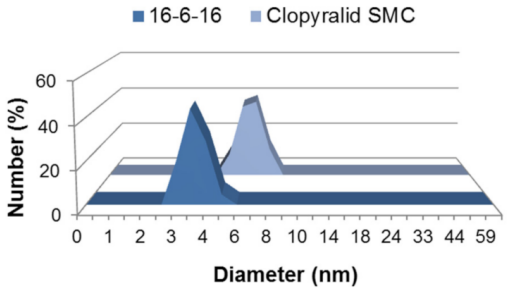


Figure 3. Hydrodynamic diameter of the 16-6-16 micelles in the presence and absence of clopyralid at 25 °C.

The formation of a complex of clopyralid with a surfactant may also be the cause of an increase in the effectiveness of the herbicidal formulation. It is known that surfactants are able to overcome cellular barriers and provide improved delivery of the bound herbicide into the plant, which we showed earlier in our Mirgorodskaya et al. study [25]. In addition, it is known that surfactants can partially dissolve the wax cuticle, improving its permeability [33,34].

3.2. Clopyralid SMC, Hacker WG, and Hacker 300 SL Caused Abnormalities in Cocklebur That Are Characteristic of Auxin-Mimetic Herbicides

Treatment with all formulations containing clopyralid caused typical effects on cocklebur—loss of turgor, stem curling, and leaf epinasty. Nine plants from each treatment were examined after the experiment to verify the most specific of the auxin-mimetic herbicides effects—abnormal growth (Figure 4). At the tips of the treated plants, growth was inhibited, and we observed clear signs of chlorosis, necrotic foci on the leaves, and a strong overgrowth of the meristem (4 mm) compared with Control plants, as well as browning and necrotization, especially in the main parenchyma. At the same time, in Control plants, the formation of meristems occurred anatomically correctly, as all tissues had a bright green color. The diameter of the stem in its upper zone was 2.5 mm.

The most developed axillary buds of each plant were analyzed. In Control plants, the nodes were anatomically correct, with tissues of a bright green color, and there were small, closed axillary buds in the leaf axils. The length of the largest leaves of the axillary bud varied from 2–3 mm, while the width of the buds was 1.5–2 mm (Figure 4).

In all treated plants, growth of nodes, browning, and necrosis of tissues in axillary buds were observed; buds with obvious signs of growth were observed in leaf axils. The length of the largest leaves of the axillary bud in the treated plants varied from 2 to 5 mm, and the width of the bud was from 2.5 to 5 mm, which is much larger than the Control plants (Figure 4).

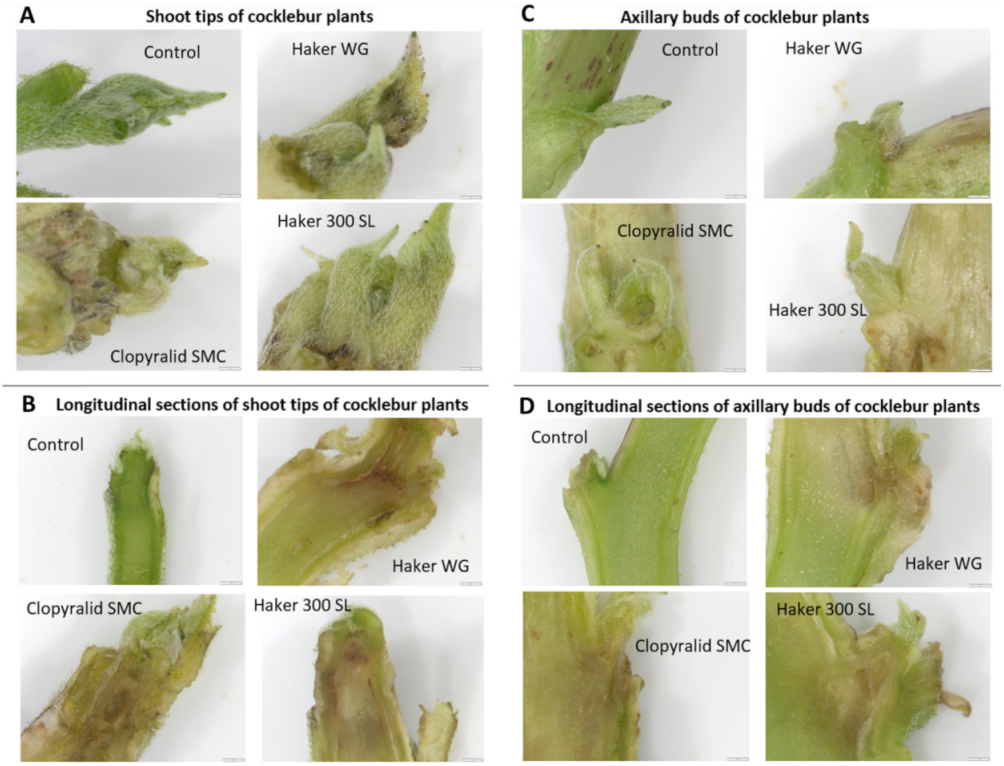


Figure 4. Characteristic abnormalities in cocklebur caused by clopyralid, observed on the 12–15th day after the herbicide application. (**A**) Shoot tips and (**B**) longitudinal sections thereof; (**C**) axillary buds and (**D**) longitudinal sections thereof. For each treatment, a series of images of nine randomly selected plants was made, the images were visually (by eye) ranked from the smallest damage to the largest, and the middle (fifth) image from each series was used for this figure.

Thus, the treatment of cocklebur plants with all formulations of clopyralid led to the expected damage; however, it was not possible to fix significant differences in the effect of various forms of the herbicide on growth points.

3.3. Clopyralid SMC Has the Highest Herbicidal Effect Based on the Changes in Morphological Parameters of Cocklebur

3.3.1. Leaf Angle Decreased with All Formulations, but the Effect of Clopyralid Was Maintained throughout the Experiment Only with the Clopyralid SMC

Under the action of herbicidal formulations, the incline of cocklebur leaves changed from an orientation of an average of 50° from the vertical (most of the foliage is slightly inclined to the ground) to 32–40° (an even greater inclination of the foliage to the ground). The most severe leaf lowering occurred in plants treated with the Clopyralid SMC (Figure 5). Hacker 300 SL was the next most efficient and Hacker WG was the least efficient. When considering the entire time series of data, all groups of plants differed statistically significantly from each other—the p-value for Control and Hacker WG was 0.01; for Hacker WG and Hacker 300 SL, it was less than 0.0001; and for other pairs of groups, the p-value was less than 10^{-6}. The difference in leaf angle values at individual time points were statistically significant between all groups on days 5–6. Before these days, the difference was not significant for the Hacker WG and Hacker 300 SL groups and the Hacker 300 SL and Clopyralid SMC groups. After day 6, the difference was not significant for the Control and Hacker WG groups and the Control and Hacker 300 SL groups.

The effect of Hacker WG developed within one day, while the effect of the Clopyralid SMC and Hacker 300 SL continued to develop for several more days. After day five of herbicide treatment, both Hacker formulations stopped reducing the leaf angle and the leaves began to return to their normal state. At the same time, the Clopyralid SMC showed

a more stable effect—the leaf angle remained at 14–15° below the normal position until the end of the observation (Figure 5).

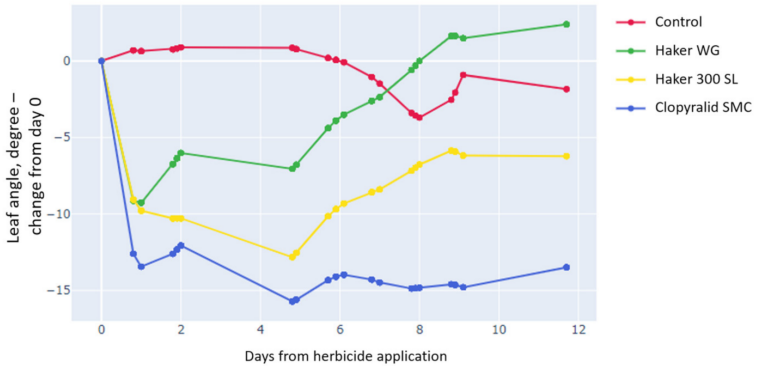


Figure 5. Dynamics of changes in the leaf angle of the cocklebur treated with various herbicides and Control plants.

3.3.2. Clopyralid SMC Shows the Strongest Effect on Plant Height, Leaf Area, and Digital Biomass

Clopyralid action leads to a decrease in turgor and wilting of herbicide-treated plants, accompanied by a decrease in their observed height and a change in leaf shape. This was reflected in the determined values of plant height and leaf surface area (leaf area). These parameters started to decrease immediately after the treatment with clopyralid formulations, reaching a minimum on the first day after treatment (Figure 6A,B). For Hacker WG and Hacker 300 SL, the plants began to recover by these parameters on the second day, while for the Clopyralid SMC, no recovery was observed until the end of the study.

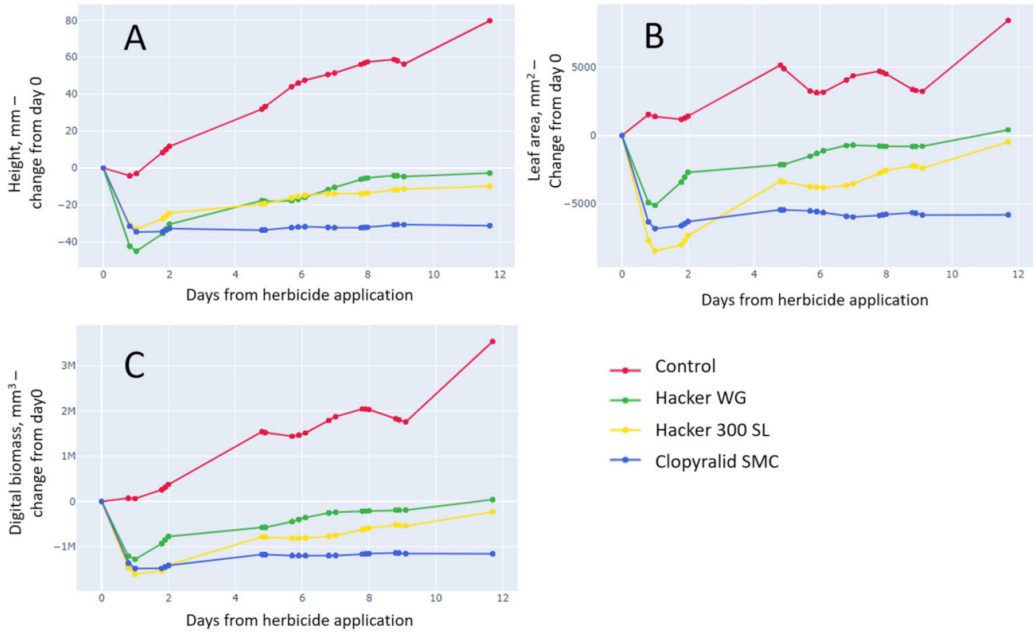


Figure 6. Dynamics of changes in the leaf angle of the cocklebur treated with various herbicides and Control plants. (**A**) Average height of the plants after subtraction of the initial values at the time of the herbicide treatment. (**B**) Average leaf area of the plants after subtraction of the initial values at the time of the herbicide treatment. (**C**) Average digital biomass of the plants after subtraction of the initial values at the time of the herbicide treatment.

Digital biomass is calculated as the product of height and leaf area (Figure 6C). The trends for all treatments for digital biomass are similar to the trends for height and leaf

area. All groups are statistically significantly different from each other when all time points are considered ($p < 0.0001$). The differences between groups are significant for all time points from the sixth day for all treatments except Hacker WG and Hacker 300 SL. Thus, based on digital biomass, the Clopyralid SMC demonstrates a more durable and efficient herbicide effect compared with both Hacker formulations. TraitFinder measurements of leaf area and digital biomass are shown to correlate well with real values determined using the traditional manual approach. The correlation coefficients of the measured and actual leaf area are usually less than one (each 1 cm^2 of actual leaf area is measured as 0.2–0.6 cm^2). The strength of correlations is high with determination coefficients (R^2) of 0.86–0.96 [35,36]. For this study, as in most other studies, the relative values of measured leaf area and digital biomass are completely sufficient.

3.4. Clopyralid SMC Has the Highest Herbicidal Effect Based on the Changes of Spectral Parameters of Cocklebur

Plant pigments play a key role in the life of plants. Chlorophyll is responsible for photosynthesis, while carotenoids and flavonoids provide photoprotection and are involved in cell signaling. Spectral indices are based on the reflectivity of plants in different region of the spectrum and provide valuable information about the state of plants.

3.4.1. Normalized Differential Vegetation Index (NDVI)

NDVI is a widely used index that reflects the content of chlorophyll. The NDVI of healthy, actively growing plants is in the range of 0.5–1 and depends on the specific plant species and growing conditions. The index value for damaged or aging foliage ranges from below 0.5 down to negative values. The distribution of NDVI values by points in 3D models of scanned cocklebur plants is shown on Figure S2.

In this experiment, the average NDVI values of healthy cocklebur plants were 0.64–0.69. After herbicide treatment, the mean NDVI values began to decrease, and this decrease continued for at least two days for all formulations (Figure 7A). An analysis of the entire set of measurements showed that all groups differ statistically significantly from each other in terms of changes in the average NDVI spectral index. Analysis of the statistical significance of differences between groups at each individual measurement (time point) showed that Control plants differed significantly from plants treated with herbicide formulations from the first day. The mean NDVI values of the plants treated with the Clopyralid SMC began to differ from those of the Hacker WG and Hacker 300 SL groups from the second day. As for the differences between plants treated with Hacker WG and Hacker 300 SL, this was statistically significant only in a number of measurements in the interval between the second and sixth days after treatments, after which the significance of the differences disappeared.

Analysis of the NDVI bins showed that the proportion of healthy cocklebur foliage (bin 4) at the starting point of the experiment was 89–94% of the total foliage area. After the treatments, the proportion of healthy foliage fell by 15–20% in two days for all treatments (Figure 7B). Subsequently, the proportion of healthy foliage in plants treated with Hacker WG and Hacker 300 SL began to gradually increase. At the same time, in plants treated with the Clopyralid SMC, the proportion of healthy foliage continued to fall further and stabilized at a level of 25–30% below the initial proportion (Figure 7B).

The proportion of damaged foliage of cocklebur plants (bin 3) was initially 5–8%. As a result of the herbicide treatments, the share of such foliage increased by 15–18% on the second day for all treatments (Figure 7C). After that, the proportion of such foliage in plants treated with Hacker WG and Hacker 300 SL began to gradually decrease. At the same time, in plants treated with the Clopyralid SMC, the proportion of damaged foliage continued to grow, exceeding the initial proportion by 28% on the fifth day (Figure 7C).

In the analysis of bins, as in the case of the average NDVI, all groups differed significantly from each other when considering the entire time series.

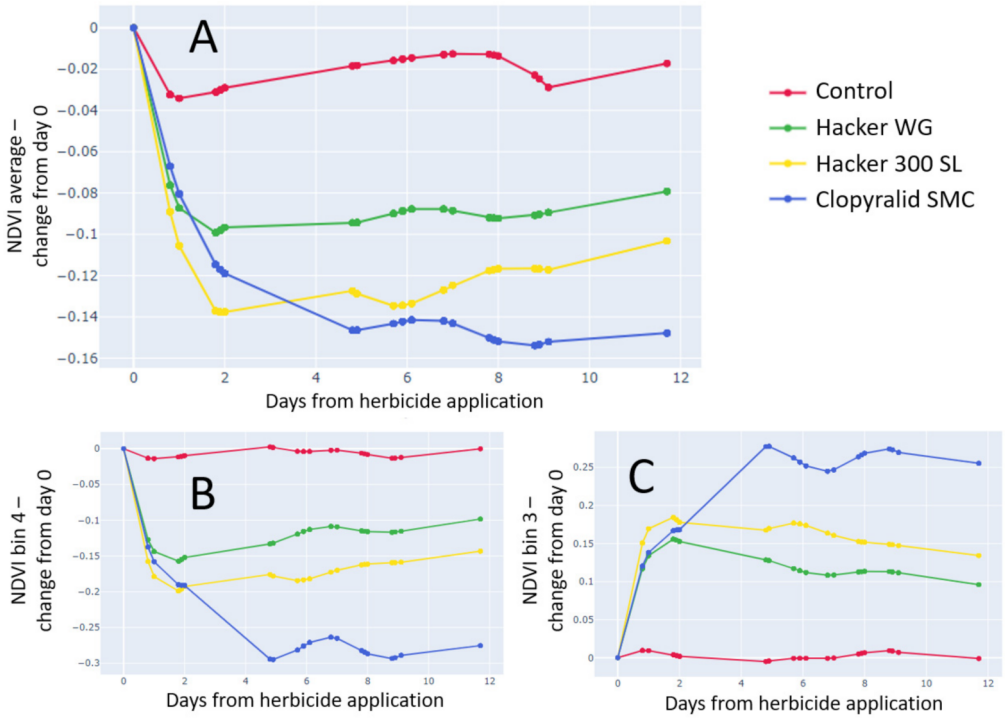


Figure 7. Dynamics of changes in NDVI (normalized differential vegetation index) of cocklebur treated with various herbicides and Control plants. (**A**) Average NDVI of the plants after subtraction of the initial values at the time of the herbicide treatment. (**B**) Fraction of leaves with NDVI in the range 0.5–1 ("bin 4"—leaves with good chlorophyll content) after subtraction of the initial values at the time of the herbicide treatment. (**C**) Fraction of leaves with NDVI in the range 0–0.5 ("bin 3"—leaves with low chlorophyll content) after subtraction of the initial values at the time of the herbicide treatment.

When comparing individual time points for average NDVI or for bins, all groups were statistically different from each other except between Hacker WG and Hacker 300 SL, where there was practically no statistical significance, and also at some time points between Hacker WG and Control.

Based on NDVI, the most effective herbicide action was shown by the Clopyralid SMC, and the least effective action by Hacker WG. The Hacker 300 SL had an intermediate efficacy.

3.4.2. Plant Senescence Reflectance Index (PSRI)

The PSRI is a spectral index based on the reflectance in the red, green, and NIR spectral regions ((Section 2 Formula (2)), and it reflects the content of chlorophyll, carotenoids and other pigments that give the leaves a yellowish and reddish hue. This index can be considered a stress indicator. The PSRI of cocklebur not treated with herbicides in our study ranged from −0.2 to 0.2. Under the action of herbicides, the PSRI of a part of the foliage increased to values from 0.2 to 0.8. Distribution of PSRI values by points in 3D models of scanned cocklebur plants is shown in Figure S3.

In our experiment, the average PSRI value of healthy cocklebur plants was about −0.01. After herbicide treatment, the mean PSRI began to increase for one day in the case of Hacker WG and up to five to six days for the Clopyralid SMC and Hacker 300 SL (Figure 8A). Considering the entire set of measurements, all groups differed statistically significantly from each other except for Hacker WG and Hacker 300 SL, which were not significantly different. Analysis of the statistical significance of differences between the groups at each individual measurement (time point) showed that the Clopyralid SMC significantly differed from Control plants and both the Hacker formulations, while Hacker

WG and Hacker 300 SL did not differ significantly. Both Hacker WG and Hacker 300 SL were significantly different to Control plants within the first six to seven days; after which, there was no difference with the Control.

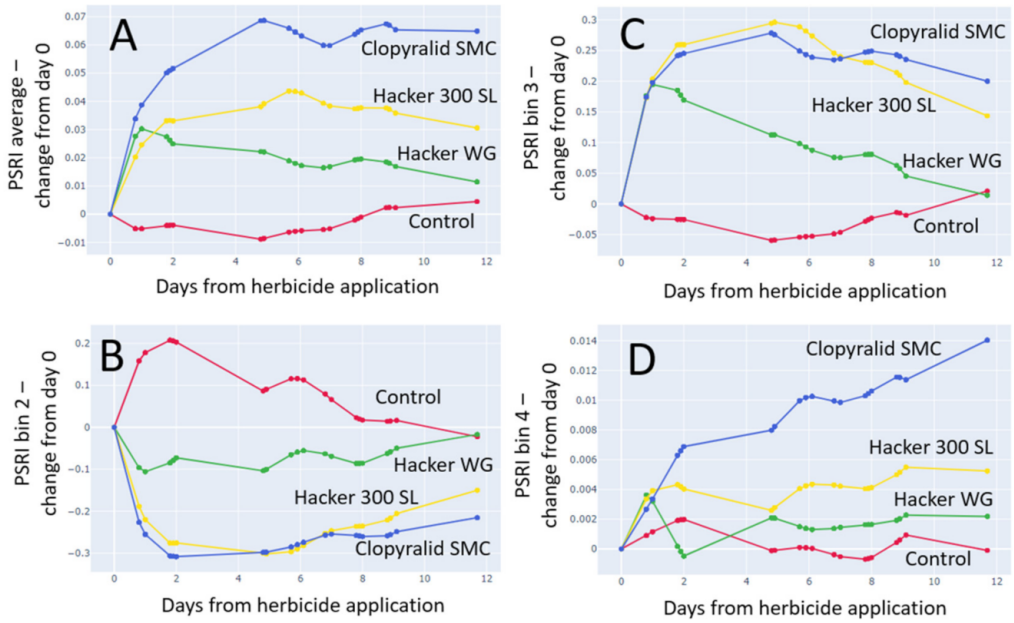


Figure 8. Dynamics of changes in the PSRI (plant senescence reflectance index) of cocklebur treated with various herbicides and Control plants. (**A**) Average PSRI of the plants after subtraction of the initial values at the time of the herbicide treatment. (**B**) Fraction of leaves with PSRI in the range −0.5 to 0 ("bin 2"—healthy leaves) after subtraction of the initial values at the time of the herbicide treatment. (**C**) Fraction of leaves with PSRI in the range 0–0.5 ("bin 3"—stressed leaves) after subtraction of the initial values at the time of the herbicide treatment. (**D**) Fraction of leaves with PSRI in the range 0.5–1 ("bin 4"—strongly damaged leaves) after subtraction of the initial values at the time of the herbicide treatment.

Analysis of bins 2–4 shows the decrease in the proportion of healthy foliage (bin 2) and the increase in the proportion of stressed (bin 3) and severely stressed (bin 4) foliage in plants treated with the herbicide formulations (Figure 8B–D). According to bin 2 and bin 3, Hacker 300 SL performs similarly to the Clopyralid SMC; however, these two formulations are very different for bin 4 (Figure 8D). For all bins, the difference between all groups is statistically significant when considering the full time series. The difference between individual time points for all bins are generally similar to the average PSRI with the exception of bin 4, where Hacker WG and Hacker 300 SL are not different from the Control.

Based on the PSRI, the Clopyralid SMC had the most effective herbicide action, followed by Hacker 300 SL, and Hacker WG showed the least effective action. Clopyralid SMC was the only formulation that caused severe leaf damage (bin 4—Figure 8D).

The summation of the data obtained allows us to state that, in practically all respects, the Clopyralid SMC has a stronger and more pronounced herbicidal effect on cocklebur plants compared with the clopyralid formulations Hacker WG and Hacker 300 SL without gemini surfactants.

Our study also shows that digital phenotyping is a very useful tool to determine the effect of herbicides on plants. The advantages of digital phenotyping are its non-invasiveness, high throughput, accuracy, and lesser impact of human error [37,38]. Even a small difference between plants can be reliably detected using this approach. With digital phenotyping, spectral and morphological manifestations of various stresses in plants, such as drought, heat stress [39], nutritional deficiencies [40], and plant diseases [41], are successfully measured. Depending on the plant, the type of stress, and the available tools for determining stress, various spectral indices have been developed, the total number

of which exceeds a hundred [42]. Usually, the determination of the effect of herbicides is carried out by calculating the survival rate of plants after treatment. However, this requires a long time of observation of the plants. In some studies, in addition to calculating the survival rate, the effect of clopyralid was also assessed by measuring the diameter of the plant rosette, measuring the digital leaf area using a handheld meter from LiCor, weighing dried plant parts [43], and measuring the root length growing from onion bulbs [44].

This study was conducted using a relatively short observation (phenotyping) period after herbicide treatment of the plants. Although, in some cases, weeds regrow a few weeks after herbicide treatment, phenotyping lasted only 12 days in this study. Our short 12-day digital phenotyping study design was based on the fact that we compared the efficacy of the new herbicide formulation with commercial formulations at the active ingredient dosage recommended for agricultural use (label dose). Even if such a dosage does not cause irreversible death of the weed, increasing the dosage of the herbicide is of no practical importance, since it is forbidden to exceed the label dose. Therefore, to compare the efficacy of herbicide formulations, a relatively short period of phenotyping of plants was chosen; on the one hand, this would allow the observation of characteristic changes caused by the herbicide, while, on the other hand, being well suited for rapid screening of the efficacy of herbicide formulations.

We have not found studies on the identification of the most sensitive spectral indices for detecting and measuring the herbicidal effect of clopyralid. A study by Bloem et al. [45] on the effect of glyphosate, a herbicide with a different mechanism of action to clopyralid, on a mixture of grasses showed that the best sensitivity for detecting herbicide action was achieved using the NDLI (normalized difference lignin index, which depends on the content of lignin), CRI-1 (carotenoid reflectance index), and PRI (photochemical reflectance index, which reflects photosynthetic activity). These indices revealed the effect of glyphosate on the third or fourth day after the application of the herbicide. NDVI revealed the effect of glyphosate only from the sixth day, and PSRI was not tested in this study [45].

In our study of the effect of clopyralid preparations, the chlorophyll-related index NDVI detected statistically significant differences between the Control group of plants and all groups of treated plants on the first day after treatment, and this difference was observed throughout the observation period (12 days). The chlorophyll- and carotenoid- sensitivity index PSRI revealed statistically significant differences between Control plants and all groups of treated plants on the first day after treatment as well. However, the difference between the two Hacker formulations and Control plants disappeared after six days, while the difference between Control and Clopyralid-SMC-treated plants persisted throughout the observation period. In contrast to the basic morphological traits (leaf area, plant height and digital biomass), which changed only on the day of herbicide treatment and then gradually returned to the initial level, the spectral indices NDVI and PSRI changed within two or, in the case of the Clopyralid SMC, up to five days. Leaf angle changes occurred within one day for the Hacker WG group and lasted up to five days for the Hacker 300 SL and Clopyralid SMC groups. Thus, in our study, the spectral indices and the leaf angle turned out to be indicators that detect the development of herbicide stress for a longer time compared with the basic morphological characteristics.

It is worth noting that the study design used in this work can be useful not only for comparing the effect of various herbicide formulations, but also for the search for herbicide-tolerant crops [46,47]. Herbicides can adversely affect the growth and yield of the crops they protect, and digital phenotyping can help identify herbicide-tolerant accessions.

4. Conclusions

In this work, it was shown that, due to non-valent interactions between the cationic gemini surfactant 16-6-16 and the herbicide clopyralid, a supramolecular complex is formed which can be used for weed control, balancing between the herbicide efficacy and the negative ecological impact. With digital phenotyping using the cocklebur plant (*Xanthium strumarium*) as a model weed, we have shown that, according to all the morphological and

spectral parameters (digital biomass, leaf area and angle, NDVI, and PSRI spectral indices), the formed supramolecular complex has the strongest and most long-lasting herbicidal action compared with other formulations of clopyralid such as Hacker WG and Hacker 300 SL. The positive effect of surfactants on the effectiveness of pesticides is primarily associated with their ability to act as carriers of biologically active substances. Due to its amphiphilic nature, 16-6-16 tends to self-assemble and forms nanosized complexes with the herbicide, thereby providing the ability to overcome cellular barriers. An additional factor responsible for the improved efficacy of the surfactant–clopyralid formulations is the improved wetting, which ensures more effective contact of the herbicide with the plant surface. The results obtained indicate significant potential of gemini surfactants as adjuvants in herbicidal formulations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13040973/s1, Figure S1: Spreading of a drop on Parafilm: 1—clopyralid 0.03%; 2—16-6-16 0.01%; 3—16-6-16 0.01% + clopyralid 0.03%; Figure S2: Distribution of NDVI (Normalized Difference Vegetation Index) values by points in 3D models of scanned cocklebur plants at the end of the experiment (day 12 from herbicide application); Figure S3: Distribution of PSRI (Plant Senescence Reflectance Index) values by points in 3D models of scanned cocklebur plants at the end of the experiment (day 12 from herbicide application).

Author Contributions: Conceptualization, L.M.N. and A.B.M.; methodology, L.M.N. and A.B.M.; validation, A.B.M., D.Y.L., M.G.D. and L.M.N.; formal analysis, R.A.K. and D.Y.L.; investigation, R.A.K., A.A.U., A.O.B. and I.A.K.; resources, L.M.N. and M.G.D.; writing—original draft preparation, A.B.M., D.Y.L. and L.M.N.; writing—review and editing, A.B.M., L.Y.Z., D.Y.L., M.G.D., L.M.N. and I.A.K.; visualization, R.A.K., D.Y.L. and A.A.U.; supervision, L.Y.Z., M.G.D. and L.M.N.; project administration, L.M.N. and M.G.D.; funding acquisition, L.M.N. and M.G.D. All authors have read and agreed to the published version of the manuscript.

Funding: Digital phenotyping was performed thanks to the support of the Ministry of Science and Higher Education of the Russian Federation and financial support under the government assignment No. #0431-2022-0008. A.B.M., R.A.K., and L.Y.Z. are grateful for financial support from the government assignment for the FRC Kazan Scientific Center of RAS.

Data Availability Statement: Not applicable.

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

References

1. Castro, M.J.L.; Ojeda, C.; Cirelli, A. Advances in surfactants for agrochemicals (Review). *Environ. Chem. Lett.* **2014**, *12*, 85–95. [CrossRef]
2. Kovalchuk, N.M.; Simmons, M.J.H. Surfactant-mediated wetting and spreading: Recent advances and applications. *Curr. Opin. Colloid Interface Sci.* **2021**, *51*, 101375. [CrossRef]
3. Dollinger, J.; Schacht, V.J.; Gaus, C.; Grant, S. Effect of surfactant application practices on the vertical transport potential of hydrophobic pesticides in agrosystems. *Chemosphere* **2018**, *209*, 78–87. [CrossRef] [PubMed]
4. Palma-Bautista, C.; Vazquez-Garcia, J.G.; Travlos, I.; Tataridas, A.; Kanatas, P.; Domínguez-Valenzuela, J.A.; De Prado, R. Effect of adjuvant on glyphosate effectiveness, retention, absorption and translocation in *Lolium rigidum* and *Conyza canadensis*. *Plants* **2020**, *9*, 297. [CrossRef] [PubMed]
5. Hu, X.; Gong, H.; Li, Z.; Ruane, S.; Liu, H.; Pambou, E.; Bawn, C.; King, S.; Ma, K.; Li, P.; et al. What happens when pesticides are solubilized in nonionic surfactant micelles. *J. Colloid Interface Sci.* **2019**, *541*, 175–182. [CrossRef]
6. Räsch, A.; Hunsche, M.; Maila, M.; Burkhardt, J.; Noga, G.; Pariyar, S. Agricultural adjuvants may impair leaf transpiration and photosynthetic activity. *Plant Physiol. Biochem.* **2018**, *132*, 229–237. [CrossRef]
7. Krogh, K.; Halling-Sørensen, B.; Mogensen, B.; Vejrup, K. Environmental properties and effects of nonionic surfactant adjuvants in pesticides: A review. *Chemosphere* **2003**, *50*, 871–901. [CrossRef]
8. Bao, Z.; Wu, Y.; Song, R.; Gao, Y.; Zhang, S.; Zhao, K.; Wu, T.; Zhang, C.; Du, F. The simple strategy to improve pesticide bioavailability and minimize environmental risk by effective and ecofriendly surfactants. *Sci. Total Environ.* **2022**, *851*, 158169. [CrossRef]

9. Hu, X.; Gong, H.; Liu, H.; Wang, X.; Wang, W.; Liao, M.; Li, Z.; Ma, K.; Li, P.; Rogers, S.; et al. Contrasting impacts of mixed nonionic surfactant micelles on plant growth in the delivery of fungicide and herbicide. *J. Colloid Interface Sci.* **2022**, *618*, 78–87. [CrossRef]
10. He, L.; Ding, L.; Li, B.; Mu, W.; Li, P.; Liu, F. Regulating droplet wetting and pinning behaviors on pathogen-modified hydrophobic surfaces: Strategies and working mechanisms. *J. Agric. Food Chem.* **2021**, *69*, 39, 11720–11732. [CrossRef]
11. Baratella, V.; Renzaglia, M.; Trinchera, A. Effect of surfactant as adjuvant for irrigation/fertigation in vegetables production: Preliminary results on lettuce. *Acta Hortic.* **2016**, *1123*, 157–163. [CrossRef]
12. Wojcieszak, M.; Krupa, B.; Syguda, A.; Walkiewicz, F.; Wilkowska, M.; Kozak, M.; Materna, K. Surface activity and phytotoxicity of morpholinium herbicidal ionic liquids. *J. Mol. Liq.* **2022**, *362*, 119750. [CrossRef]
13. Vereshchagin, A.N.; Frolov, N.A.; Egorova, K.S.; Seitkalieva, M.M.; Ananikov, V.P. Quaternary ammonium compounds (QACs) and ionic liquids (ILs) as biocides: From simple antiseptics to tunable antimicrobials. *Int. J. Mol. Sci.* **2021**, *22*, 6793. [CrossRef] [PubMed]
14. Mirgorodskaya, A.B.; Kushnazarova, R.A.; Sharonova, N.L.; Rakhmaeva, A.M.; Tyryshkina, A.A.; Kuznetsov, D.M.; Nikitin, E.N.; Zakharova, L.Y. New piperidinium surfactants with a benzyl fragment in the head group: Aggregation properties and the possibility of using to control plant pathogens. *Russ. Chem. Bull.* **2022**, *71*, 1679–1686. [CrossRef]
15. Zhou, C.; Wang, Y. Structure–activity relationship of cationic surfactants as antimicrobial agents. *Curr. Opin. Colloid Interface Sci.* **2020**, *45*, 28–43. [CrossRef]
16. Van Ginkel, C.G. Biodegradability of cationic surfactants. In *Biodegradability of Surfactants*; Karsa, D.R., Porter, M.R., Eds.; Springer: Dordrecht, The Netherlands, 1995. [CrossRef]
17. Menger, F.M.; Keiper, J.S. Gemini surfactants. *Angew. Chem. Int. Ed.* **2000**, *39*, 1906–1920. [CrossRef]
18. Ahmady, A.R.; Hosseinzadeh, P.; Solouk, A.; Akbari, S.; Szulc, A.M.; Brycki, B.E. Cationic gemini surfactant properties, its potential as a promising bioapplication candidate, and strategies for improving its biocompatibility: A review. *Adv. Colloid Interface Sci.* **2022**, *299*, 102581. [CrossRef]
19. Sharma, R.; Kamal, R.; Abdinejad, M.A.; Mahajan, R.K.; Kraatz, H.-B. Advances in the synthesis, molecular architectures and potential applications of gemini surfactants. *Adv. Colloid Interface Sci.* **2017**, *248*, 35–68. [CrossRef] [PubMed]
20. Bao, Y.; Gao, L.; Wang, F.; Ma, J. Heterocyclic cationic Gemini surfactants for efficient antibacterial, dispersion and fixation. *Process Saf. Environ. Prot.* **2022**, *159*, 168–177. [CrossRef]
21. Amerkhanova, S.K.; Voloshina, A.D.; Mirgorodskaya, A.B.; Lyubina, A.P.; Kuznetsova, D.A.; Kushnazarova, R.A.; Mikhailov, V.A.; Zakharova, L.Y. Antimicrobial properties and cytotoxic effect of imidazolium geminis with tunable hydrophobicity. *Int. J. Mol. Sci.* **2021**, *22*, 13148. [CrossRef]
22. Pisárčik, M.; Pupák, M.; Lukáč, M.; Devínsky, F.; Hubčík, L.; Bukovský, M.; Horváth, B. The Synthesis, Self-assembled structures, and microbicidal activity of cationic gemini surfactants with branched tridecyl chains. *Molecules* **2019**, *24*, 4380. [CrossRef] [PubMed]
23. Frolov, N.; Detusheva, E.; Fursova, N.; Ostashevskaya, I.; Vereshchagin, A. Microbiological evaluation of novel bis-quaternary ammonium compounds: Clinical strains, biofilms, and resistance study. *Pharmaceuticals* **2022**, *15*, 514. [CrossRef] [PubMed]
24. Marcinkowska, K.; Sobiech, Ł.; Kuliszewska, E.; Skrzypczak, G. Effect of gemini surfactants on the glyphosate efficacy. *Przem. Chem.* **2014**, *93*, 911–913.
25. Mirgorodskaya, A.B.; Kushnazarova, R.A.; Lukashenko, S.S.; Nikitin, E.N.; Sinyashin, K.O.; Nesterova, L.M.; Zakharova, L.Y. Carbamate-bearing surfactants as effective adjuvants promoted the penetration of the herbicide into the plant. *Colloids Surf. A* **2020**, *586*, 124252. [CrossRef]
26. Todd, O.E.; Figueiredo, M.R.; Morran, S.; Soni, N.; Preston, C.; Kubeš, M.F.; Napier, R.; Gaines, T.A. Gaines. Synthetic auxin herbicides: Finding the lock and key to weed resistance. *Plant Sci.* **2020**, *300*, 110631. [CrossRef] [PubMed]
27. Unger, T.A. Clopyralid. In *Pesticide Synthesis Handbook*; Elsevier: Amsterdam, The Netherlands, 1996; p. 530.
28. Boyd, N.S.; Dittmar, P.J. Impact of application time and clopyralid rate on strawberry growth and yield. *Weed Technol.* **2015**, *29*, 29821–29826. [CrossRef]
29. Bobadilla, L.; Hulting, A.; Curtis, D.; Mallory-Smith, C. Application of synthetic auxin herbicides to suppress seed viability of Italian ryegrass (*Lolium perenne* ssp *multiflorum*) in tall fescue seed production. *Weed Technol.* **2020**, *34*, 489–497. [CrossRef]
30. Mirgorodskaya, A.B.; Kudryavtseva, L.A.; Pankratov, V.A.; Lukashenko, S.S.; Rizvanova, L.Z.; Konovalov, A.I. Gemini alkylammonium surfactants: Aggregation properties and catalytic activity. *Russ. J. Gen. Chem.* **2006**, *76*, 1625–1631. [CrossRef]
31. Gao, X.; Wang, D.; Jiang, Z.; Li, X.; Chen, G. Effect of adjuvants on the wetting behaviors of bifenthrin droplets on tea leaves. *Appl. Sci.* **2022**, *12*, 4217. [CrossRef]
32. Song, Y.; Huang, Q.; Huang, G.; Liu, M.; Cao, L.; Li, F.; Zhao, P.; Cao, C. The Effects of adjuvants on the wetting and deposition of insecticide solutions on hydrophobic wheat leaves. *Agronomy* **2022**, *12*, 2148. [CrossRef]
33. Schreiber, L. Review of sorption and diffusion of lipophilic molecules in cuticular waxes and the effects of accelerators on solute mobilities. *J. Exp. Bot.* **2006**, *57*, 2515–2523. [CrossRef] [PubMed]
34. Cardoso Damato, T.; Carrasco, L.D.M.; Carmona-Ribeiro, A.M.; Luiz, R.V.; Godoy, R.; Petri, D.F.S. The interactions between surfactants and the epicuticular wax on soybean or weed leaves: Maximal crop protection with minimal wax solubilization. *Crop. Prot.* **2017**, *91*, 57–65. [CrossRef]

35. Vadez, V.; Kholová, J.; Hummel, G.; Zhokhavets, U.; Gupta, S.K.; Hash, C.T. LeasyScan: A novel concept combining 3D imaging and lysimetry for high-throughput phenotyping of traits controlling plant water budget. *J. Exp. Bot.* **2015**, *66*, 5581–5593. [CrossRef] [PubMed]
36. Kjaer, K.H.; Ottosen, C.-O. 3D Laser Triangulation for Plant Phenotyping in Challenging Environments. *Sensors* **2015**, *15*, 13533–13547. [CrossRef] [PubMed]
37. Walter, A.; Liebisch, F.; Hund, A. Plant phenotyping: From bean weighing to image analysis. *Plant Methods* **2015**, *11*, 14. [CrossRef]
38. Yang, W.; Feng, H.; Zhang, X.; Zhang, J.; Doonan, J.H.; Batchelor, W.D.; Xiong, L.; Yan, J. Crop Phenomics and High-Throughput Phenotyping: Past Decades, Current Challenges, and Future Perspectives. *Mol. Plant* **2020**, *13*, 187–214. [CrossRef]
39. Hein, N.T.; Ciampitti, I.A.; Jagadish, S.K. Bottlenecks and opportunities in field-based high-throughput phenotyping for heat and drought stress. *J. Exp. Bot.* **2021**, *72*, 5102–5116. [CrossRef]
40. Li, D.; Li, C.; Yao, Y.; Li, M.; Liu, L. Modern imaging techniques in plant nutrition analysis: A review. *Comput. Electron. Agric.* **2020**, *174*, 105459. [CrossRef]
41. Tanner, F.; Tonn, S.; de Wit, J.; Van den Ackerveken, G.; Berger, B.; Plett, D. Sensor-based phenotyping of above-ground plant-pathogen interactions. *Plant Methods* **2022**, *18*, 35. [CrossRef]
42. Xue, J.; Su, B. Significant Remote Sensing Vegetation Indices: A Review of Developments and Applications. *J. Sens.* **2017**, *2017*, 1353691. [CrossRef]
43. Ghanizadeh, H.; Li, F.; He, L.; Harrington, K.C. Characterization of clopyralid resistance in lawn burweed (*Soliva sessilis*). *PLoS ONE* **2021**, *16*, e0253934. [CrossRef] [PubMed]
44. Amaç, E.; Liman, R. Cytotoxic and genotoxic effects of clopyralid herbicide on *Allium cepa* roots. *Environ. Sci. Pollut Res.* **2021**, *28*, 48450–48458. [CrossRef]
45. Bloem, E.; Gerighausen, H.; Chen, X.; Schnug, E. The Potential of Spectral Measurements for Identifying Glyphosate Application to Agricultural Fields. *Agronomy* **2020**, *10*, 1409. [CrossRef]
46. Ranjan, P.N.; Ram, C.J.; Anurag, T.; Nilesh, J.; Kumar, P.B.; Suresh, Y.; Rahul, K. Breeding for herbicide tolerance in crops: A review. *Res. J. Biotechnol.* **2020**, *15*, 4.
47. Abou-Khater, L.; Maalouf, F.; Patil, S.B.; Balech, R.; Nacouzi, D.; Rubiales, D.; Kumar, S. Identification of tolerance to metribuzin and imazethapyr herbicides in faba bean. *Crop Sci.* **2021**, *61*, 2593–2611. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.