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Interaction of Organic Solvents with the Epicuticular Wax Layer of Wheat Leaves

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ABSTRACT: After foliar application, compounds that are not absorbed into leaves can be removed from the leaf surface by dipping or rinsing in dilutions of organic solvents in water. However, interactions between solvent mixtures and the epicuticular wax layer have received little attention, and information on potential physical and chemical intactness of the plant surface following application of solvents is limited. In this study, wheat leaves were dipped in organic solvents at different dilutions with water, and the major component of the leaf epicuticular wax layer, 1-octacosanol, was analyzed to assess damage to the wax layer. Dipping leaves in dilutions of organic solvent higher than 60% by volume resulted in only negligible or low levels of 1-octacosanol extraction, while no 1-octacosanol was detected in any mixtures containing less than 40% organic solvent. Furthermore, analysis of leaf surfaces by scanning electron microscopy showed structural intactness of the epicuticular wax layer when organic solvent mixtures were used. Therefore, our results demonstrate that the epicuticular wax layer of wheat leaves is not altered physically or chemically by organic solvent solutions up to 40% by volume. These findings validate the use of solvent washing procedures to assess unabsorbed compounds on wheat leaf surfaces.

KEYWORDS: *Acetonitrile, acetone, methanol, ethanol, isopropanol, organic solvent, 1-octacosanol, solubility, epicuticular wax, wheat*

INTRODUCTION

The plant epicuticular wax layer is known to function in keeping water within plant tissues,[1] serving as a barrier to pathogens and insects,[2,3] mitigating ultraviolet (UV) radiation,[4] and in special cases, acting as a lubricated surface to trap insects within the girdles of pitcher plants for digestion.[5] To better understand this diverse set of functions, the chemical compositions of epicuticular wax layers of many plant species have been characterized, including rose,[6] cranberry,[7] orange,[8] cherry laurel,[9] and wheat.[10]

In general, cuticular waxes have been classified either as "intracuticular" when they are embedded within the cutin and/or cutan matrix or "epicuticular" when they are present as a wax layer deposited on the surface of the cutin polymer.[11−14] Regardless of the species, the majority of wax layers consists of a complex mixture of fatty acids, alcohols, esters, and alkanes with variable chain lengths.[6−10] The compositions of the chemicals within the wax layers and the thickness and three-dimensional structure of the layers contribute to the species-specific properties of these wax layers.[6] It can be reasonably assumed that a thicker wax layer may better prevent leaves from dehydration and may affect the rate of absorption of solutes across cuticles; however, cases have been identified where other factors, such as chemical compositions of wax layers, the morphological structure, and interactions with the surrounding environment, can be more important than merely a thicker cuticle wax layer.[6,13,15]

The penetration of various organic compounds into the epicuticular wax layer of plants has been investigated.[16−22] In these studies, dipping or washing plant leaves in the various solutions was used as a procedure to remove unabsorbed compound residues remaining on the leaf surface. However, in a number of studies, quantitative analysis of foliar uptake of compounds was based on the assumption that washing its surface with solutions only removes compounds on the plant surface but does not remove compounds that have penetrated into the wax layer or inside the plant tissues.[23−25] Currently, there is no scientific evidence that the assumption is correct. Therefore, it is important to understand the interaction of the epicuticular wax layer and the common organic solvent mixtures that are often applied to wash compounds off of plant surfaces.

In this study, 1-octacosanol, the most abundant primary alcohol in the epicuticular wax layer of wheat leaves,[10,26] was selected as an internal marker molecule. The removal of 1-octacosanol from the epicuticular wax layer of wheat leaves by five organic solvents and dilutions of the solvents with water was quantitatively examined to assess damage to the wax layer and, therefore, to address the potential for removing foliar-applied compounds from the epicuticular wax layer when these solutions are used. The qualitative microscopic analyses were also performed to investigate the effect of the solutions on wax layer integrity and morphology.

MATERIALS AND METHODS

Dipping Solutions. Five organic solvents (methanol, ethanol, isopropanol, acetonitrile, and acetone) and water/organic solvent mixtures [80:20, 60:40, 40:60, or 20:80, v/v (volume/volume)] were used. In addition to the solutions, chloroform was used as a positive standard because of its higher wax-dissolving power, while water, in which epicuticular wax layer components are insoluble, was chosen as a negative standard.[6−8,13,19] The solvent mixtures were freshly prepared immediately prior to the development of experiments.

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Table 1. Concentration of 1-Octacosanol Recovered in Various Solutions at Different Concentrations of Water and Organic Solvent after Dipping Wheat Leaves in the Solutions for 1, 2, or 3 min[a]

		concentration of 1-octacosanol (μg/g of leaf fresh weight)		
percent of water/organic solvent by volume	organic solvent used	1 min	2 min	3 min
	chloroform	2564.7 ± 315.8	2197.3 ± 206.5	2743.2 ± 557.0
0:100	methanol	19.0 ± 1.0	35.0 ± 2.8	48.2 ± 0.3
	ethanol	22.7 ± 3.8	36.2 ± 2.5	55.3 ± 2.3
	isopropanol	179.3 ± 23.3	305.3 ± 27.2	384.0 ± 43.8
	acetone	124.2 ± 19.8	267.8 ± 38.3	426.8 ± 8.5
	acetonitrile	0.7 ± 0.1	1.0 ± 0.3	3.2 ± 1.0
20:80	methanol	trace[b]	trace	trace
	ethanol	0.5 ± 0.2	0.8 ± 0.2	1.0 ± 0.2
	isopropanol	8.0 ± 1.7	15.3 ± 2.0	27.0 ± 7.2
	acetone	1.2 ± 0.3	2.3 ± 0.5	3.7 ± 0.7
	acetonitrile	trace	trace	trace
40:60	methanol	nd[c]	nd	nd
	ethanol	trace	trace	trace
	isopropanol	trace	trace	trace
	acetone	trace	trace	trace
	acetonitrile	trace	trace	trace
60:40	methanol	nd	nd	nd
	ethanol	nd	nd	nd
	isopropanol	nd	nd	nd
	acetone	nd	nd	nd
	acetonitrile	nd	nd	nd
80:20	methanol	nd	nd	nd
	ethanol	nd	nd	nd
	isopropanol	nd	nd	nd
	acetone	nd	nd	nd
	acetonitrile	nd	nd	nd

[a]Values are shown as means ± standard deviations of triplicates. [b]Trace indicates the presence of 1-octacosanol in samples where its peak area was below 250 counts. [c]nd = not detected.

Leaf Dipping and Sampling Procedures. 'Yuma' wheat plants were grown from seeds in 50% mineral soil and 50% soil-less Metro mix in plastic pots in a greenhouse for 7 days. Primary leaves of the plants were cut at their base, and four leaves were carefully dipped into 10 mL of organic solvent or solvent mixture prepared as described above in a 16 × 100 mm culture test tube (Fisherbrand, Pittsburgh, PA) for 1, 2, or 3 min, without soaking the cut base of the leaves. After removal of leaves from the tube, the remaining solution was subsequently evaporated using a Savant Speed Vac concentrator or under N_2 gas and the resulting residue was dissolved in 1 mL of chloroform. Unless otherwise indicated, all of the experiments were carried out with three replications for every solution.

1-Octacosanol Solubility. A large volume of 1-octacosanol (Sigma-Aldrich, St. Louis, MO) was prepared in chloroform at a concentration of 1 mg/mL, and a 200 μL aliquot was divided into multiple tubes and dried under N_2 gas. A total of 1 mL of various solvents prepared as described above was added to the test tubes, and the samples were sonicated for 10 min. After sonication, the solutions were passed through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter (Millipore, Florham, NJ) to remove any insoluble particles, and dried. The dried residues were dissolved in 1 mL of chloroform.

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis. Prior to GC/MS analysis, 300 μL of bis-*N*,*N*-(trimethylsilyl)-trifluoroacetamide (BSTFA, Acros Organics, Fair Lawn, NJ) was added to the samples in 1 mL of chloroform and the samples were then incubated for 20 min at 80 °C, as previously described.[27] Derivatized 1-octacosanol in the samples was analyzed with Finnigan Trace GC equipped with Polaris Q MS (Thermo Scientific, Waltham, MA), using a DB-1701 column (30 m × 0.25 mm inner diameter and 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA). The oven temperature was initially held at 100 °C for 2 min, linearly increased to 280 °C for 9 min, and then held at 280 °C for 16 min. Helium was used as the carrier gas at a constant pressure of 200 kPa. A 2 μL injection of the derivatized sample resulted in a peak corresponding to 1-octacosanol, which eluted at 17.0 min and produced as a molecular ion at m/z 467 after sample injection, and a calibration curve constructed from a series of derivatized 1-octacosonal at various dilutions was used to quantify 1-octacosanol at the targeted ion. The limit of detection was 0.2 ng, which gave an area of approximately 250 counts from the integrated extracted ion chromatogram, and the area of a peak exhibiting 250 counts or less was considered "trace".

Microscopy. Primary leaves were cut from wheat plants, and individual leaves were dipped in different pure organic solvents, a dilution series of acetone in water by volume (80, 60, 40, or 20%) or water for 3 min. Leaf samples were washed with water immediately after dipping treatments and kept in a 15 mL falcon tube containing water until preparation for scanning electron microscopy (SEM). A 1 cm section of each leaf was collected from the approximate middle of the leaf. These pieces were mounted on carbon tape on 25 mm diameter aluminum planchets (Ted Pella, Redding, CA), and a piece of copper tape was applied across the tops and bottoms of the leaves to hold the leaves flat during air drying. The mounted specimens were left at room temperature for 24−48 h to air dry. The samples were sputter-coated (Emitech SC7620, West Sussex, U.K.) with Au/Pd for 120 s at 15 cm distance from the target. The samples were imaged in high-vacuum mode in Hitachi TM-1000 SEM (Tokyo, Japan). Images were contrast-enhanced with the FIJI version of NIH ImageJ, and figure panels were created with GIMP (version 2.8).

Leaf Injury Assessment. A plant grown in a pot was inverted, and the primary leaf was carefully dipped into the dipping solution in a 15 mL falcon tube for 3 min, without soaking the axil of the plant. The leaf was immediately rinsed with water and transferred to and

maintained in a greenhouse. The plants were watered once a day by root irrigation. Any visual damage of the leaves was monitored, and the plants were photographed for 1 week.

RESULTS AND DISCUSSION

Removal of 1-Octacosanol in Epicuticular Wax on Wheat Leaves by Various Organic Solvent Solutions. In this experiment, wheat leaves were dipped in each of five organic solvents, acetonitrile, acetone, methanol, ethanol, and isopropanol, and water/organic solvent mixtures at variable concentrations (20:80, 40:60, 60:40, and 80:20, v/v) for 3 min, and any 1-octacosanol washed off in these solutions was quantified. Chloroform removed about 2.5 mg of 1-octacosanol from a 1 g leaf within 1 min, but the removal of 1-octacosanol did not increase with extended dipping time up to 3 min (Table 1), indicating that the extraction of 1-octacosanol from leaf waxes by chloroform was complete within 1 min. As expected, no 1-octacosanol was detected in water, which was used as a negative control solution throughout this study (data not shown).

Unlike chloroform, dipping in the pure organic solvents tested in this study progressively removed 1-octacosanol for 3 min (Table 1). The results of each of the washings across all solvent concentrations showed a large degree of variance in 1-octacosanol removal and a decrease with progressive dilutions with water of the pure organic solvents. The initial removal of 1-octacosanol in isopropanol or acetone for 1 min was higher than that of methanol or ethanol, and the lowest recovery of 1-octacosanol was achieved with acetonitrile; however, the extractable amount of 1-octacosanol in each organic solvent was much lower than that in chloroform, indicating a limited removal of 1-octacosanol by the solvent. Considering that 1-octacosonal comprises 66% of the total weight of the epicuticular wax layer on wheat leaves and also determines the shape of the wax structure,[10,26] our results suggest that methanol, ethanol, and acetonitrile have less effect on properties of the epicuticular wax layer compared to acetone and isopropanol.

The removal of 1-octacosanol was sharply reduced in the progressively increasing dilutions with water (Table 1). 1-Octacosanol recovered in 80% acetone or isopropanol in water by volume was significantly lower compared to the corresponding pure organic solvent. The significant reduction by the addition of 20% water was also observed with other solvents, where only a very low or trace amount of 1-octacosanol was detected in 80% methanol, ethanol, and acetonitrile up to 3 min. Subsequent dilutions in water containing 60% organic solvent washed off only negligible or no 1-octacosanol across all time measurements, and no 1-octacosanol was detected in the dilutions of 40% or less organic solvent concentration. The results indicate that the addition of water to organic solvents can substantially attenuate their capabilities of removing 1-octacosanol, suggesting that water is a major contributing factor to determine the interaction of the solutions with the epicuticular wax layer. This can further suggest that other water-miscible organic solvents may not be able to remove 1-octacosanol if solvent mixtures containing at least 60% water are used as washing solution.

Although a threshold for the water-soluble solvent concentration at which no 1-octacosanol is removed was noted in this study, it was still questionable whether 1-octacosanol could be detected in the 40 and 20% organic solvents if a longer time of dipping was applied. Therefore, leaves were dipped in the 40 and 20% organic solvent mixtures in water up to 24 h, and 1-octacosanol in the mixtures was analyzed. We found that 1-octacosonal was not detectable in all 40 and 20% solvent mixtures even after the 24 h dipping (data not shown). Overall, our results show that mixtures containing as high as 40% organic solvent in water by volume do not remove 1-octacosanol from the epicuticular wax layer of wheat leaves.

Solubility of 1-Octacosanol in Organic Solvent Solutions. The sharp decline in the amount of 1-octacosanol removed with increasing dilutions of the solvent mixtures (Table 1) led us to investigate whether the solubility of 1-octacosanol in the mixtures is a contributing factor to this decrease. The solubility of pure 1-octacosanol was highest in isopropanol, among all of the organic solvents used in this study, but was significantly lower (only 1.8% of 1-octacosanol was dissolved) compared to that dissolved by chloroform (Figure 1). A much lower amount of 1-octacosanol was

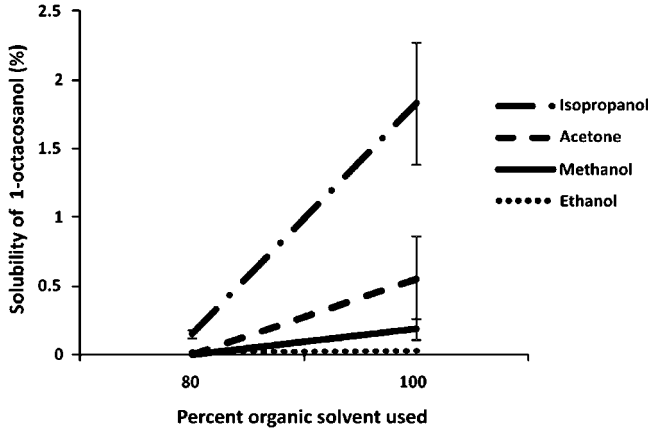

Figure 1. Solubility of 1-octacosanol in various organic solvents. The solubility of 1-octacosanol in chloroform was set to 100%. Values are shown as means ± standard deviations of triplicates.

solubilized in acetone, followed by methanol and ethanol (Figure 1), while no 1-octacosanol was detected in acetonitrile (data not shown). The results were not surprising, because Hoerr et al. reported poor solubility of long-chain aliphatic primary alcohols in polar organic solvents,[28] similar to our results. When the water content in the organic solvent mixtures increased to 20% by volume, the solubility of 1-octacosanol in the mixtures significantly decreased below 0.2% (Figure 1). Additionally, no 1-octacosanol was detected in all of the solutions containing organic solvents below 60% by volume (data not shown), demonstrating that the solubility of 1-octacosanol becomes very limited with the increase of water and the compound becomes insoluble when the water volume exceeds 40% of the solvent mixtures. It was noted that there was a trend between the extractability of 1-octacosanol in the cuticular wax layer by the organic solvents and the solubility of pure 1-octacosanol in the same solvents; the higher extractability and solubility of 1-octacosanol were achieved in isopropanol and acetone, followed by methanol, ethanol, and acetonitrile. These results suggest that the differential solubility of 1-octacosanol in different organic solvents is a contributing factor that can explain the differential removal of 1-octacosanol in the cuticular wax layer by these solvents.

SEM Investigation of Wheat Leaves Dipped in Organic Solvent Solutions. It was important to investigate

whether epicuticular crystal structures were modified in relation to the partial extraction of 1-octacosanol by organic solvents, as shown in Table 1. The surface structures of leaves subjected to dipping in organic solvents did not differ from those of the untreated leaf (panels B–F of Figure 2), except for acetone,

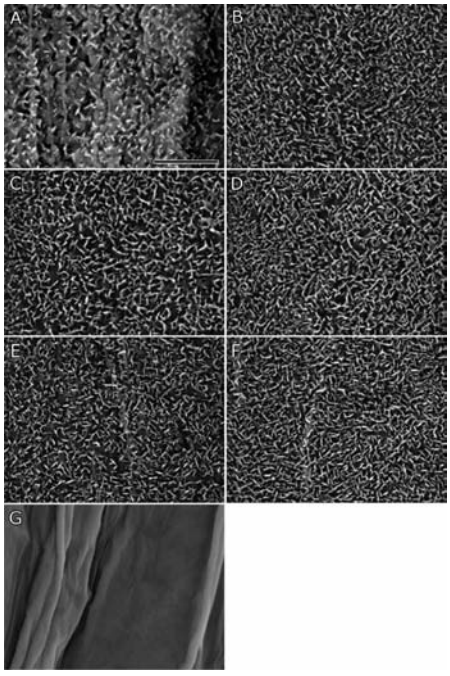

Figure 2. SEM images of the abaxial surface of wheat leaves dipped in different solvents for 3 min: (A) acetone, (B) acetonitrile, (C) ethanol, (D) isopropanol, (E) methanol, (F) untreated (water; negative control), and (G) chloroform (all epicuticular wax removed; positive control). Scale bar = 5 μm.

where a degree of melting of wax crystals was seen (Figure 2A). On the contrary, a complete removal of epicuticular wax crystals was clearly visible when wheat leaves were dipped in chloroform (Figure 2G). These results indicate that the polar organic solvents, except for acetone, do not alter the structural arrangement of epicuticular waxes, despite the partial removal of 1-octacosanol. The unaltered wax structures by pure organic solvents also suggest that water–organic solvent mixtures do not likely cause a structural change of wax layers; thus, a further evaluation on the wax layer modification by the solvent mixtures was not pursued.

Considering that 1-octacosanol in leaf samples was similarly extracted in isopropanol and acetone (Table 1) and the intrinsic solubility of 1-octacosanol was even higher in isopropanol compared to acetone (Figure 1), the dissolution of wax crystals of wheat leaves by acetone shown in Figure 2 was surprising. The unexpected melting of epicuticular wax crystals by acetone led to the investigation of the potential damage of the layer in a dilution series of acetone in water. The occurrence of disturbed structures of the leaf waxes by acetone was not observed when solvent mixtures were used (Figure 3). Overall, our results show that acetone is more destructive to the epicuticular wax layer than other organic solvents used in this study, even though the loss of 1-octacosanol in the wax layer by acetone was marginal. Thus, this suggests that, although differential solubility of 1-octacosanol in different organic solvents can explain differential removal of 1-octacosanol in the cuticular wax layer by these solvents, intrinsic solubility of 1-

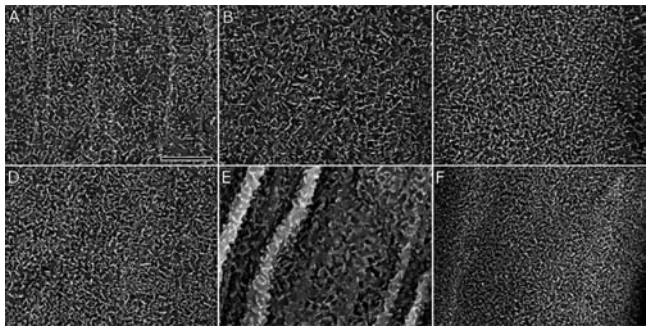

Figure 3. SEM images of the abaxial surface of wheat leaves dipped in pure acetone or a dilution series of acetone in water for 3 min: (A) 80% water/20% acetone, (B) 60% water/40% acetone, (C) 40% water/60% acetone, (D) 20% water/80% acetone, (E) 100% acetone, and (F) untreated (water; negative control). Scale bar = 5 μm.

octacosanol in these solvents alone does not explain whether the epicuticular wax will be intact. Furthermore, the intactness of the wax layer in the acetone mixture with 20% or more water indicates that the disruption of the wax layer by acetone can be significantly avoided by adding a relatively small volume of water, suggesting the important role of the water in the interaction of the organic solvent with the epicuticular wax layer.

Injury of Wheat Leaves by Organic Solvent Solutions. A partial removal of up to 10% of total 1-octacosanol in wheat leaves by organic solvents as well as a degree of dissolution of epicuticular wax by acetone, as described above (Table 1 and Figures 2 and 3), raised the question of whether these slight compositional and structural changes affect leaf survival. Therefore, potential injury to wheat leaves by the solutions was examined. In this experiment, primary leaves of plants were dipped in the solutions for 3 min and physiological changes of treated leaves were monitored over the course of 1 week.

When primary leaves were dipped in chloroform, they immediately wilted upon being removed from the solvent, but no major defects on the leaves were visible upon dipping them in isopropanol and acetone (Figure 4). However, within 2 h, primary leaves dipped in acetone began to show considerable damage, similar to the symptom of leaves dipped in chloroform, whereas only a minor yellowing on the leaf blade was observed with leaves dipped in isopropanol. The initial damage to the leaf surface by acetone and isopropanol visually remained the same at 24 h after application (Figure 4), without any progressive injury up to 1 week (data not shown). On the contrary to pure acetone and isopropanol, no visual sign of damage to leaves was detectable when dipped in solvent mixtures consisting of lower than 80% acetone or isopropanol (Figure 4). In addition, no damage by other pure organic solvents, including methanol, ethanol, and acetonitrile, as well as solvent mixtures with water, was noticeable for 1 week after dipping (data not shown).

Given that the removal of 1-octacosanol by acetone and isopropanol was similar (Table 1), if the partial removal of 1-octacosanol by the solvents was the only factor responsible for the injury, a similar degree of injury would have been expected. However, the greater dissolution of wax crystals by acetone compared to isopropanol (Figure 2) suggests that structural disintegration of the wax layer can significantly affect leaf physiology, as demonstrated by the severe damage to leaves done by acetone compared to isopropanol (Figure 4). Although

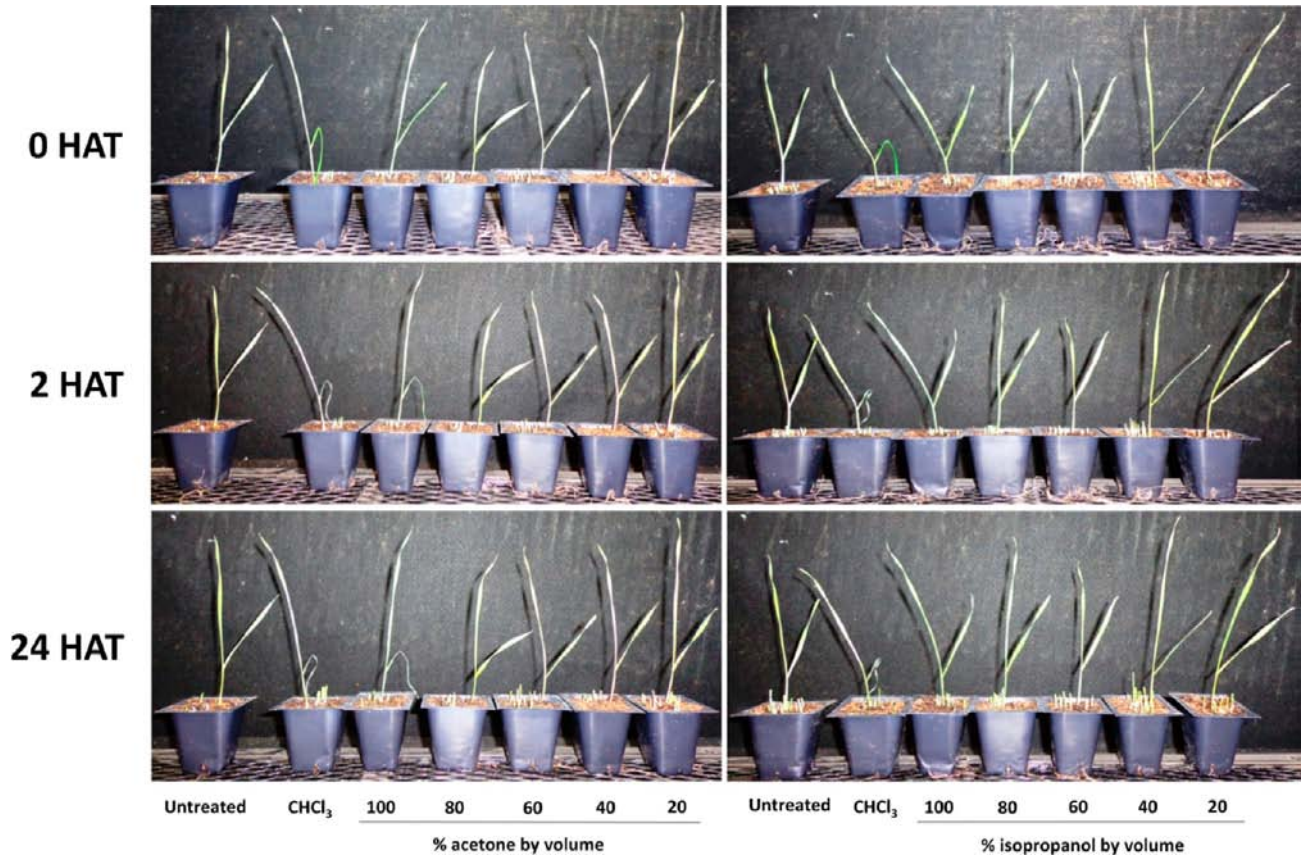

Figure 4. Images of wheat plants at 0, 2, and 24 h after dipping treatment (HAT). An individual primary leaf of a plant in each pot was dipped in different solutions for 3 min (from left to right: untreated; 100% chloroform, $CHCl_3$; and 100, 80, 60, 40, and 20% acetone or isopropanol in water by volume).

the damage caused by acetone could be the result of other unknown factors, such as a faster diffusion of acetone into leaf tissue and subsequent lysis of cellular membranes, information on the interaction of organic solvents with the cellular components of leaf tissue is limited. Nonetheless, these results indicate that, when the wax layer is intact, even with the loss of such a small quantity of 1-octacosanol from the wax, the leaves can survive, but once the layer is disorganized with the same loss, leaves are eventually damaged.

This investigation also demonstrates that the addition of as low as 20% water into acetone, the most destructive weak organic solvent to wax integrity shown in this study, completely prevented any leaf damage, pointing to the ability of water in the organic solvent mixtures to mitigate the destructive nature of the solvent. In this regard, a threshold dilution of organic solvent in water, which does not disrupt the wax layer either chemically or physically, can be established, and this can enable us to assess unabsorbed chemicals on the leaf surface separated from those sequestered within the wax layer after foliar application of chemicals of interest. Our findings suggest that any organic mixtures containing up to 40% organic solvent by volume can be safely applied to retrieve unabsorbed compound residues on wheat leaf surfaces. The use of mixtures of water with any organic solvents could provide a flexibility of selecting organic solvents depending upon solubility and stability of the compounds in the solvents, particularly for lipophilic compounds that do not dissolve in water. Additionally, considering that dipping leaves in chloroform removes more waxes than rinsing leaves with chloroform for the same amount of time,[20] it is likely that organic mixtures containing up to 40% organic solvent by volume can also be applied to remove unabsorbed compound residues without disrupting the epicuticular wax layer in the case of rinsing.

In conclusion, we described the capabilities of five organic solvents and solvent dilutions with water for removing 1-octacosanol in the epicuticular wax layer of wheat leaves and showed that 1-octacosanol was not removed when leaves were dipped in organic solvent mixtures containing at least 60% water. These findings were consistent across all solvents, regardless of intrinsic 1-octacosanol solubility in pure solvents, illustrating a protective effect of water in the mixtures. Microscopic observations showed that the epicuticular wax layer of wheat leaves was not modified following treatment with the solvent mixtures, supporting the intactness of the wax layer. Taken together, our results demonstrate that water−organic solvent mixtures containing 40% or lower organic solvent do not chemically or physically disrupt the epicuticular wax layer. Our new findings on interactions of organic solvents with the epicuticular wax layer can provide a rationale for potential applications of certain solvent-washing procedures to remove unabsorbed compounds on wheat leaf surfaces without damaging or degrading the epicuticular wax layer.

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Notes

The authors declare no competing financial interest.

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