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Leaf Cuticle Can Contribute to Non-Host Resistance to Poplar Leaf Rust

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Abstract: The plant leaf cuticle is a chemically complex but largely waxy outer shell that limits water loss and also prevents some pathogens from gaining access to internal mesophyll. Rust fungi are obligate parasites, and most bypass the cuticle by thigmotropically locating stomata, growing through the stomatal openings, and then parasitizing mesophyll cells with haustoria. It is thought that even non-hosts of a given rust fungus do not resist until their mesophyll cells are contacted in this way. In other words, it is thought that the cuticle plays no role in non-host resistance. Here, we tested the hypothesis that poplar leaf cuticles might contribute to non-host resistance to rust fungi by chemically impeding the germination and growth of urediniosporelings of *Melampsora larici-populina.* Following an initial survey in China of the resistance of 36 genotypes of various species and interspecific hybrids of *Populus* to *M. larici-populina,* we selected three genotypes for the initial test of hypothesis: (1) A *Populus purdomii* genotype that is fully susceptible; (2) a *Populus deltoides* cv. 'I-69' that is incompletely resistant (i.e., a resistant host); and (3) a *Populus tomentosa* genotype that is a non-host to *M. larici-populina*. Urediniospores were assayed for germination in extracts of the cuticles of the three genotypes. Germination was most reduced by the *P. tomentosa* non-host cuticular extracts that also reduced the growth of germ tubes to 36 times less than that in controls or in the extract of the susceptible *P. purdomii*. Four cuticular components were identified as putative defense compounds given greater concentrations in *P. tomentosa* than in *P. purdomii*: Aucubin, hexakis(trimethylsilyl) ether, catechol, 7,9-Di-tert-buty l-1-oxaspiro (4,5) deca-6, 9-diene-2,8-dione and trifluoroacetamide. These four compounds were then tested, and they reduced urediniospore germination and uredinial density in inoculations of normally susceptible *P. purdomii* with *Melampsora larici-populina*. Thus, the cuticle of *P. tomentosa* can contribute to pre-haustorial, non-host resistance to *M. larici-populina.*

Keywords: Plant defense; rust fungi; cutin; thigmotropism; leaf wetness

1. Introduction

Leaf rust fungi can cause considerable damage to the natural populations and plantings of poplars (cottonwoods or *Populus* species). These *Melampsora* species are heteroecious in that they produce their uredinia, telia and basidia on *Populus*, whereas their spermogonia and aecia form mostly on conifers, such as species of larch or *Larix*. There are at least 20 *Melampsora* species reported in China on poplars [1]. *Melampsora larici-populina* is the most important in that it is widely distributed in Eurasia. It has also been introduced in many other parts of the world. It affects poplars in two major sections of the genus *Populus*: *Tacamahaca* (the balsam poplars) and *Aigeiros* (the black poplars).

Poplar rust urediniospores are like all other urediniospores in that, after having been dispersed on the wind, they will sometimes land on leaves and then germinate during the periods of surface wetness of cuticles. The sporelings of most rust fungi must thigmotropically find their way to stomata [2].

After entering stomata and growing down into the mesophyll, rust fungi form haustorial mother cells. These may then produce parasitic haustoria in living host mesophyll cells. Successful parasitism enables the development of rust reproductive structures such as uredinia. Uredinia grow and force their way back up through host tissue, and then they erupt through host cuticles to release spores capable of dispersal and repeated rounds of infection. Successful infection thus involves not only the initial entry into host tissue but also the establishment of parasitic haustoria and reproduction. The latter definition of successful infection also applies to a second group of those few rust fungi that directly penetrate through the cuticle. For example, *Phakopsora pachyrhizi* is known to directly penetrate the cuticle and underlying epidermis and then establish successful haustoria in mesophyll cells [3], which is then followed by an eruption of uredinia. A third group of rust fungi, including the rust fungus of this study, takes both infection routes: Thigmotropic location and entry of stomata, as well as direct penetration [1,4]. However, when members of this third group directly penetrate the cuticle and epidermal cells of hosts, they do not successfully establish haustoria and uredinia [4]; these rusts are as reliant on stomatal location and entry as the first-mentioned group.

All rust fungi have limited host ranges. Non-host resistance is that which is expressed by plants towards the pathogens of other plants. In other words, plants outside the host range of a given rust species are non-hosts for those rusts. On the cuticles of both hosts and non-hosts, rust fungi of the first and third groups must locate and enter stomata, through which they grow to enter substomatal space. In susceptible hosts, haustoria are established in mesophyll cells, and the parasitic relationship develops further. However, in non-hosts, rust fungi fail to establish parasitic haustoria in mesophyll cells, and they thus fail to develop and reproduce. In other words, no new uredinia are produced in non-hosts. Non-host resistance to rust fungi is thus described as 'pre-haustorial,' and that term mostly refers to the stoppage of the infection process after the penetration of stomata but prior to the establishment of a first haustorium [5]. However, it is possible that some non-hosts resist by interfering with the thigmotropic location of stomata, perhaps by simply slowing the germination and growth of sporelings.

The chemically complex cuticle could easily contribute to non-host resistance to rusts by slowing the thigmotropic progress of urediniosporelings on the leaf surface. With long-chain fatty acids, alkanes, primary and/or secondary alcohols, aldehydes, ketones, esters, triterpenes, sterols, and flavonoids, among others [6–8], there are many cuticular candidates for such a role. The Eurasian poplar leaf rust fungus *Melampsora larici-populina* is a member of the third group of rust fungi in that its direct penetration of the cuticle does not result in successful infection [4]; it must enter stomata and then successfully infect susceptible poplar species and hybrids in the *Tacamahaca* and *Aigeiros* sections of the genus *Populus* [4]. The species of *Populus* in the *Populus* section (aspens and white poplars including *Populus tremuloides*, *Populus alba*, *Populus tremula*, and many Asian species including *Populus tomentosa*) are reported to be non-hosts to *M. larici-populina* [9–11]. Host resistance is, of course, also seen in the *Tacamahaca* and *Aigeiros* sections. In this study, we first verified the susceptibility and resistance of 36 genotypes of various species and interspecific hybrids of *Populus* to *M. larici-populina* in China. We then tested the pre-haustorial hypothesis that the leaf cuticle of *P. tomentosa*, a non-host, slows the germination and growth of *M. larici-populina*. By doing so, stomatal entry might be prevented. The particular components of the cuticle of *P. tomentosa* were finally tested to see if they could be responsible for the effects of the whole cuticular extract.

2. Materials and Methods

2.1. Resistance to M. larici-populina of 36 Clones of Poplar Species and Hybrids

Susceptibility and host and non-host resistance were evaluated in September 2018 at the Weihe River poplar experimental station (E108.084162. N34.274189) in China. In September, poplar rust due to *M. larici-populina* has fully developed and disease severity is maximal [3]. Included at the sectional level (Supplementary Table S1) in the planting were clones or genotypes representing 11 intersectional

hybrids of *Tacamahaca* and *Aigeiros*, 16 clones representing pure *Aigeiros*, 8 for the *Tacamahaca* section, and 1 *Populus* (i.e., *P. tomentosa*) clone. In total, there were 375 trees in the planting, and all had been planted 8–11 years prior to data collection. Rust severity was scored as the number of uredinia per square centimeter using a standard area transparent circle. This was achieved by counting uredinia within 1 cm^2, with five squares per leaf and five leaves toward the bottom of the canopy of each tree.

2.2. Poplar Materials for Experiments

Populus tomentosa, a non-host of *M. larici-populina*, *Populus deltoides* cv. 'I-69', a strongly resistant host, and *P. purdomii*, a susceptible host, for all experiments were sampled in Yangling, on the campus of Northwest A&F University. Leaves from these poplars were collected in early spring and lateJuly; they were treated at once for cuticle wax extraction. *Populus purdomii* was grown in a Yangling greenhouse at 25 °C, 70%–80% relative humidity (RH), and 16 h photoperiod (2500 Lux at leaf level) for in planta experiments.

2.3. Cuticle Extraction

Poplar leaf samples were collected in May and October. Four leaves (the 5^{th}–8^{th} youngest) of the three clones (*Populus tomentosa*, *Populus purdomii* and *P. deltoides* cv. 'I-69') were detached and measured for leaf area using a leaf area meter (Licor 3000, LICOR, Inc., Lincoln, NE, USA). After recording leaf area (cm^2), leaves were dried and immediately extracted. Freshly harvested leaves were immersed in 20 mL of redistilled chloroform solution in a 50 mL beaker for 30 s and gently shaken a few times, then washed with 5 mL of chloroform twice. The total of 30 mL was preserved as an extracted cuticle solution [6].

2.4. Urediniospore Germination in Leaf Cuticle Extracts

The cuticle extractions of May and October were merged together and concentrated in an evaporator at 40 °C until the volume left was 1–1.5 mL. These solutions were transferred to 2 mL centrifuge tubes for the further evaporation of chloroform until a dry cuticle pellet was obtained. One milliliter of 0.01% tween-80 was added to each sample tube to dissolve cuticle pellets, and that was used to assay urediniospores on a glass for germination (in a dark chamber at 25 °C and above 90% RH for 48 h); all urediniospores were from the experimental station. The germination rate and length of germ tubes were recorded, and the inhibitory effects of the cuticle extracts was calculated as follows: GIR = [(CK − EG)/CK] × 100%, in which GIR was the germination inhibition rate, CK was the control (0.01% tween-80 water solution), and EG was a treatment with a particular cuticle extract. Each cuticle extract experiment was repeated three times, and at least 200 urediniospores were assayed each time [12].

2.5. Identification of Cuticle Compounds

Cuticle chloroform solutions were concentrated in a Thermostat machine (Eppendorf Inc., Hamburg, Germany) to the volume of 0.5–1 mL at 40 °C. They were then transferred to Gas Chromatography (GC) tubes for further evaporation till cuticle pellets were then harvested. A 30 µL mixed solution of pyridine and silylation reagent (BSTFA) with the volume ratio 1:1 was dropped into the tube for reaction at 70 °C for 60 min. After that, approximately 1 mL of chloroform was added into the GC tube again to stop reactions. Products were dried by a constant nitrogen dryer at 70 °C and thus made ready for gas chromatography-mass spectrometry (GC-MS) [13–15]. GC-MS reaction parameters included a length of 12 m and a diameter of 0.2 mm of the GC capillary column; carrier gas, helium at 1 uL/min; inlet temperature 280 °C; split ratio, 2:1; Flame Ionization Detector (FID) detector temperature, 320 °C; process of column temperature, 50 °C 2 min, then temperature increase to 240 °C at the rate of 20 °C·min^{-1}, 2 min at 240 °C, then temperature rise to 320 °C at the rate of 1.5 °C·min^{-1}, 2 min at 320 °C; and a 10 µL C_{27} alkane (0.2 µg/µL) as an internal reference [15].

Cuticle compounds were identified by comparing their mass spectra to those of known compounds within the National Institute of Standards and Technology database (NIST, https://www.nist.gov/). Their relative content (percentage of each compound within the total cuticle content) and the absolute content of cuticles (total cuticle (μg) of per leaf area (cm^2)) were estimated by using Labsolution software [14]. Stepwise linear regression and principal component analysis (PCA) of SPSS22.0 were used to analyze the relationship between cuticle components and non-host resistance, and then the candidate components were selected for urediniospore germination assays. Only the relative content over 0.1% components were counted.

2.6. Experiments with Cuticular Components

All candidate components were purchased from J&K Scientific (Beijing, China). Components included aucubin, hexakis (trimethylsilyl) ether, catechol (N,O-Bis-(trimethylsilyl)valine (2TMS) derivative), 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6, 9-diene-2,8-dione, trifluoroacetamide (2TMS derivative), and 9-Octadecenamide, (Z)), and they were mixed with 0.01% of Tween-80 and reached final concentrations of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 mg/mL, respectively. In the in vitro assay, urediniospores of 1×10^5 /mL were suspended in the solution of different concentrations of components to determine the germination as described above. Germination rate and tube length were recorded at 24 h incubation. In the in planta assay, the seedlings of *P. purdomii* were sprayed first solutions on both sides of the youngest fifth or sixth leaves. After that, a suspension in water of urediniospores (1×10^5/mL) was sprayed onto abaxial surfaces for few minutes. The inoculated seedlings were kept in the dark at 25 °C, 100% RH chamber for 24 h, then moved to the greenhouse at 25 °C, 70%–80% RH, and 16 h photoperiod with 2500 Lux [12]. After 11 days, uredinial densities were determined as above.

3. Results

3.1. Rust Incidence and Severity in the Field

The 36 poplar clones were divided into three groups on the basis of resistance to *M. larici-populina* as measured by incidence and severity. The first group comprised only one clone: The rust-free, non-host *P. tomentosa*. The second was characterized by varying levels of host resistance and included *P. deltoides* cv. 'I-69', *P.* x *euramericana* and their hybrids, and a total of 14 clones. The third group was comprised of the remaining 21 genotypes, all of which were fully susceptible to rust: All *Tacamahaca* species and the hybrids of the *Tacamahaca* and *Aigeiros* sections. As already suggested by various authors and as summarized by international databases [16], *P. tomentosa* was free of rust in an area dominated by *M. larici-populina* (Supplementary Figure S1). The ramets of clones were always grouped together, as their uredinial densities varied little (Figure 1, Supplementary Table S1).

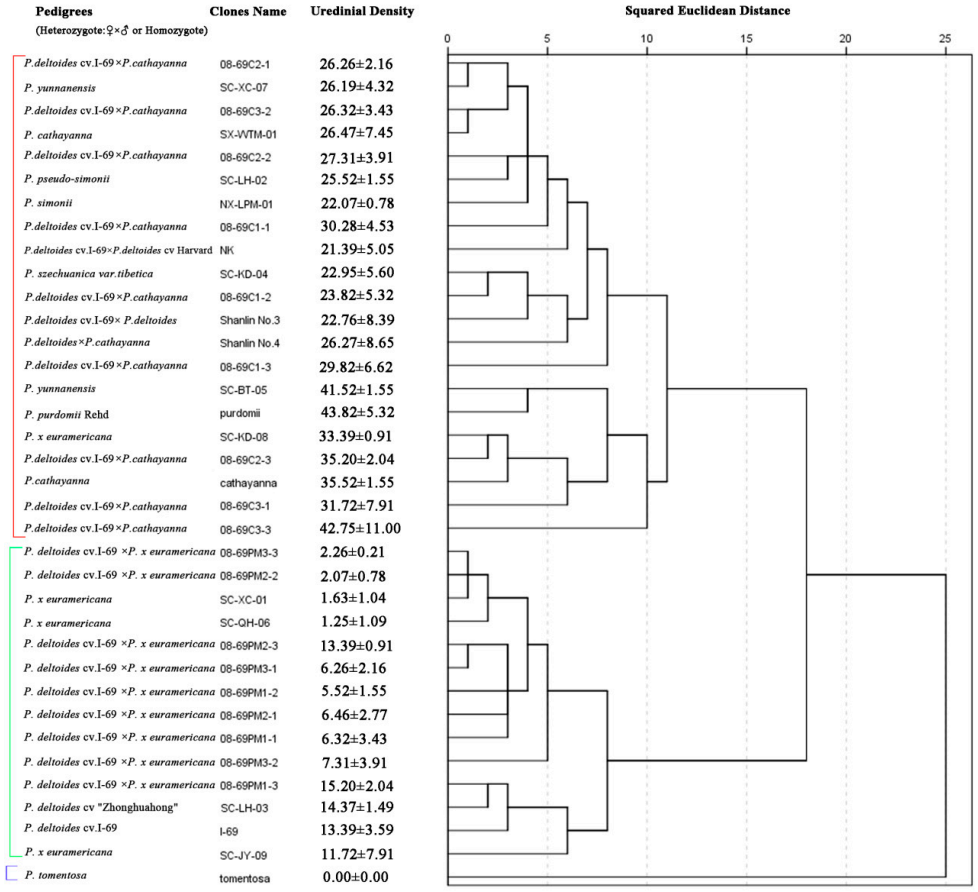


Figure 1. Dendrogram of 36 poplar clones clustered according to uredinial densities recorded in Weihe River poplar experimental station, China, in September 2018. *Melampsora larici-populina* was the sole rust.

3.2. Urediniospore Germination in Leaf Cuticle Extracts

Rust survey results allowed us to test our hypothesis with the following: Susceptible *P. purdomii* from the *Tacamahaca* section, host-resistant *P. deltoides* cv. 'I-69' from the *Aigeiros* section, and *P. tomentosa* that, like other species in the *Populus* section, displayed non-host resistance to *M. larici-populina*. First, urediniospores germinated in the aqueous cuticle extract of *P. purdomii* at a level that did not differ significantly from that of the controls ($p > 0.05$). However, the cuticle extracts of *P. tomentosa* and *P. deltoides* cv. I-69 did significantly decrease the germination rate and the growth of germ tubes (Table 1). The length of germ tubes in the *P. tomentosa* extract was approximately 36 times shorter than in the controls and the susceptible *P. purdomii* extracts. The germination inhibition of the *P. tomentosa* cuticle was 87.4%, versus 55.2% in the cuticle of *P. deltoides* cv. I-69.

Table 1. Germination rates and germ tube lengths of urediniospores in cuticle extracts.

	P. tomentosa	*P. deltoides* cv. 'I-69'	*P. purdomii*	Control
Urediniospore germination (%)	7.8 ± 2.3 a	27.8 ± 8.5 b	58.2 ± 4.8 c	62.0 ± 5.4 c
Tube length (μm)	7.7 ± 5.6 a	62.3 ± 29.9 b	278.0 ± 126.7 c	282.5 ± 113.3 c

Note: Urediniospores were assayed in solutions of the extracted cuticles of the three species in 1 mL of 0.01% tween-80. Means were distinguished with Duncan's multiple range test at $p < 0.05$. '±' is followed by standard errors.

3.3. Chemical Composition of the Leaf Cuticle

The GC-MS analysis identified 39 cuticular compounds from each of *P. tomentosa* and *P. deltoides* cv. I-69, although 39.6% of the compounds in *P. deltoides* and 4.54% in *P. tomentosa* remained unidentified. Still, their relative content was minor compared to the 39 identified compounds. The *Populus purdomii* cuticle had 37 of these compounds; in its case, only 10.3% of compounds failed to be recognized (Supplementary Figure S2). No significant difference in the identified cuticle compositions among these three taxa was found. However, a significant difference existed in the absolute and relative contents of certain cuticular components, calculated by the values of internal reference C_{27} alkane contents. *Populus tomentosa*, the non-host, had the highest absolute quantity of cuticle per leaf area: 5.6628 μg/cm^2. Susceptible *P. purdomii* had, in contrast, only 1.6667 μg/cm^2. *Populus deltoides* cv. 'I-69' was not only intermediate in resistance but also intermediate in this respect, with an absolute content of 4.1371 μg/cm^2.

With respect to the absolute and relative contents of each cuticular component, differences among susceptible hosts, non-hosts and resistant hosts were seen for alkanes, alcohols, fatty acids, esters, phenols, ethers, ketones, and amide substances (Figure 2A). For example, alcohols were the biggest group of constituents (Figure 2B), but they were relatively richer in *P. purdomii* and thus unlikely to be responsible for the non-host resistance of *P. tomentosa*.

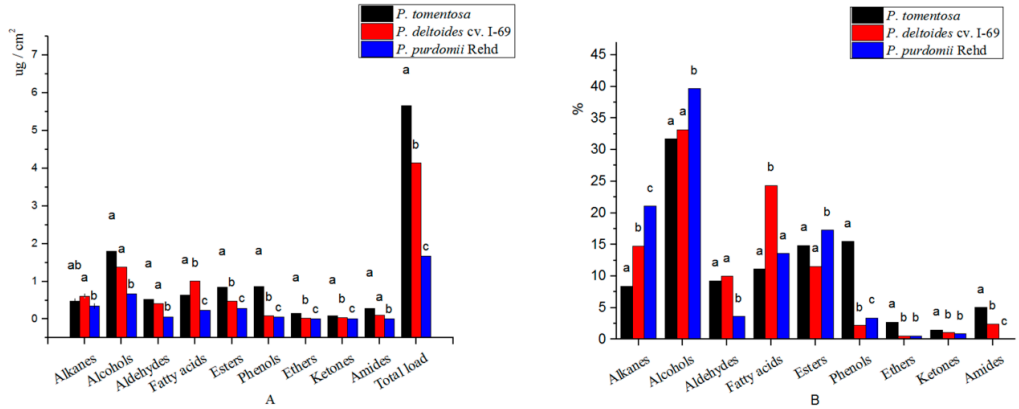


Figure 2. Absolute (**A**) and relative (**B**) quantities of cuticular compounds in *Populus purdomii*, *Populus deltoides* and *Populus tomentosa* susceptible hosts, resistant hosts and non-hosts, respectively, to *M. larici-populina*. Note: 'Absolute content' is the total quantity (μg) per unit square centimeter of leaf. The 'relative content' is the ratio of each cuticle component divided by the total cuticle content. Error bars are standard errors of the means. Significance at $p < 0.05$ is indicated by different letters above bars (Duncan's multiple range test).

The principal component analysis (PCA) based on the relative contents of nine groups of cuticle constituents revealed the two PCs explained 88.98% of the total variance; Figure 3. The first dimension (54.43% of total variance) helped to differentiate aldehydes, esters and fatty acids from the other cuticle components, while the second dimension (34.55% of total variance) classified alkanes, alcohols in one category and ethers, phenols, ketones, amides in another (Figure 3); the latter were relatively rich in *P. tomentosa* and *P. deltoides*.

Regression of cuticular compounds on urediniospore germination showed that, after excluding collinearity, two of the ten initial groups remained (i.e., alkanes and cuticle total loads) (Table 2). The regression equation was as following: Germination rate = 61.555 + (1.012A) − (20.758B), where A = alkanes, B = ethers, R = 0.952, and δ = 0.000). The coefficient of ethers was negative, as overall they reduced the germination of urediniospores. In contrast, the coefficient of alkanes was positive, as they increased germination. Thus, combining with PCA above, the ethers group (including ethers, phenols, ketones, and amides) was selected for further inhibition tests. The other group (alkanes and alcohols

rich in susceptible *P. purdomii*) showed a negative relationship with rust resistance, whereas the third group, including aldehydes, esters and fatty acids, exhibited an ambiguous role in rust resistance.

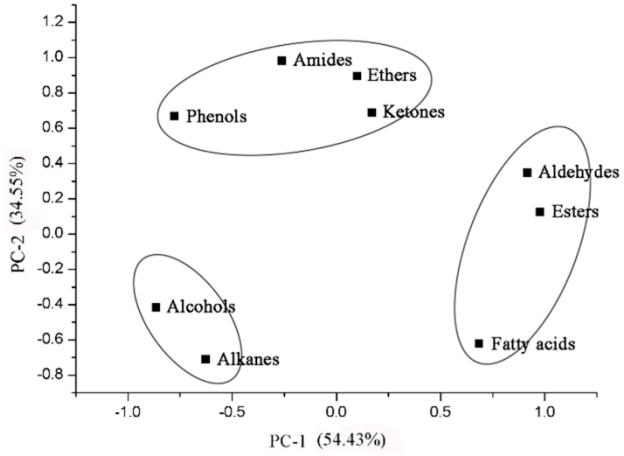


Figure 3. The principle components analysis (PCA) load diagram accorded to the relative concentrations of cuticle compounds in poplar leaves.

Table 2. Linear regression analysis between the urediniospore germination and concentration of cuticle compounds.

Model	Coefficients		Collinearity Statistics		R	Residual (δ)
	B	VIF	Eigenvalue	Condition Index		
Constant	61.555		1.728	1.000		
Alkanes	1.012	1.004	0.086	5.809	0.952	0.000 [a]
Total load	−20.758	1.213	0.272	2.522		

Note: The concentration of cuticle compounds as an independent variable and urediniospores germination rate as a dependent variable for regression analysis. VIF < 10, Eigenvalue > 0, and condition index < 30 revealed there was no collinearity. [a] was abbreviation of approximately.

3.4. Identification of Cuticle Components to Be Used in Further Assays

The relative concentrations of each cuticular compound are displayed in Figure 4. The alkanes of C_8, C_{15}, C_{18}, C_{28}, C_{35}, C_{44} and the alcohols of C_9, C_{22}, and C_{33} were richer in susceptible *P. purdomii*. On the other hand, the aldehydes of C_{28}, the esters of C_8, C_{14}, C_{17}, and C_{44}, and the fatty acids of C_{10}, C_{13}, C_{15}, C_{25}, and C_{27} were richer in the cuticles of non-host *P. tomentosa* or resistant host *P. deltoides*. The ketones of C_{13} and the amides of C_{18} appeared to contribute to the strong rust resistance of *P. deltoides*, as did the C_{33} ether. The phenols of C_{12} and C_{15}, the ketones of C_{14}, and C_{17}, and the amides of C_8 and C_{18} all appeared to contribute to the non-host resistance of *P. tomentosa*. However, these components were not all simultaneously found in *P. deltoides* and *P. tomentosa*. In this study, therefore, the top five candidates for further assays (Supplementary Table S2) were selected (Table 3).

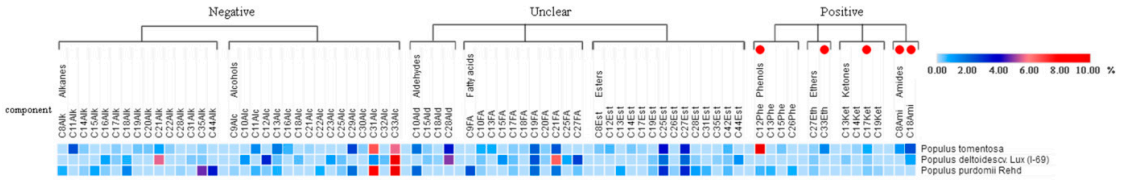
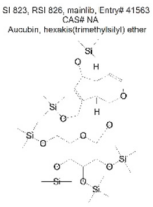
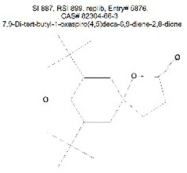
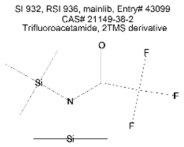
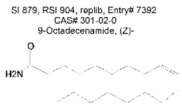


Figure 4. Relative concentrations of all cuticle compounds of the three poplar taxa varying in resistance to *M. larici-populina*. Note: Higher concentrations are in darker blue and then red. The red dots were the compounds selected for further assays and fully explained in Table 3.

Table 3. The candidate cuticular compounds most positively associated with rust resistance.

Variety	Carbon Number	Compound Name	Molecular Formula	Chemical Structure	SI	RSI
Ether	C_{33}	Aucubin, hexakis(trimethylsilyl) ether	C33H70O9Si6		823	826
Phenol	C_{12}	Catechol, 2TMS derivative	$C_{12}H_{22}O_2Si_2$		931	937
Ketone	C_{17}	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6, 9-diene-2,8-dione	$C_{17}H_{24}O_3$		929	934
Amide	C_8	Trifluoroacetamide, 2TMS derivative	C8H18F3NOSi2		921	922
	C_{18}	9-Octadecenamide, (Z)	C18H35NO		885	911.5

Note: Only library matches with a direct matching factor (SI) and a reverse search matching (RSI) higher than 700 were included.

These five compounds were class III according to Cramer rules, which implied these candidates had toxicity risks. However, they were negative for genotoxic carcinogenicity according to the Benigni–Bossa rule base. The chemical toxicities of both the C_{33} ether (aucubin, hexakis (trimethylsilyl) ether) and the C_{12} phenol (catechol, 2TMS derivative) were derived from the chemical structure of aromatic rings with complex substituents. Lactone fused to another ring, or 5- or 6-membered a,b-unsaturated lactone and heterocyclicity were the main sources of the chemical toxicity of the C_{17} ketone (7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6, 9-diene-2,8-dione). Fluoride ions played a role in the toxicity of the C_8 amide (trifluoroacetamide, 2TMS derivative). The toxicity of the C_{18} amide (9-Octadecenamide, Z-) was likely based on a long open chain.

3.5. Assays of Candidate Cuticle Components

Urediniospores in the control mostly germinated from 2 to 16 hpi (hours post-inoculation). Control germination was 50.12% at 8 hpi and stopped increasing at 24 hpi. Four candidate components, including aucubin-hexakis (trimethylsilyl) ether, catechol (2TMS derivative), 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, trifluoroacetamide (2TMS derivative), in order, decreased urediospore germination (Figure 5A and Supplementary Figure S3A) and uredinial densities in planta (Figure 5B); 9-Octadecenamide, (Z) was a little less effective (Figure 5B and Supplementary Figure S3B).

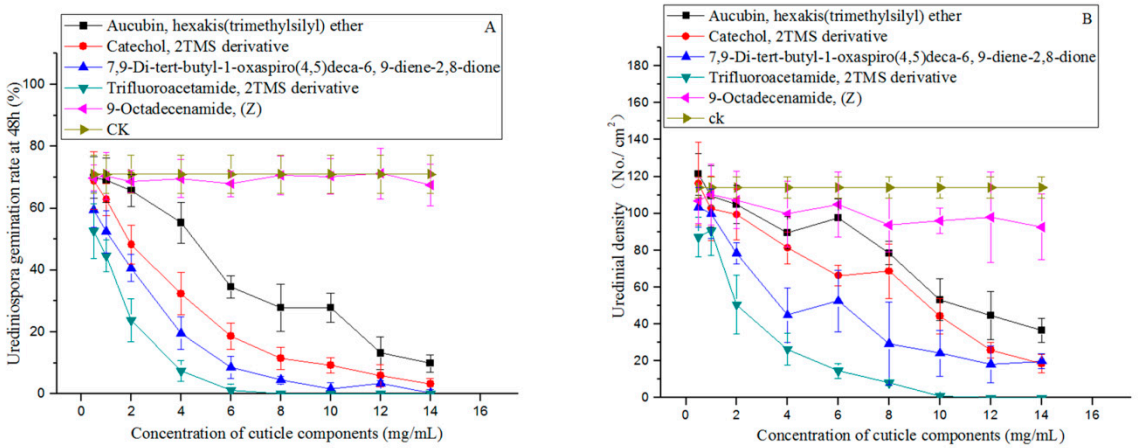


Figure 5. The effects of candidate compounds on the urediniospore germination and the number of pustules per square centimeter on leaves of *P. purdomii*. (**A**): The germination rate of urediniospores incubated in different cuticle components at 48 h. (**B**): Uredinial density on the leaves 11 days after inoculation with *Melampsora larici-populina*. The values represent the means of the replicates. The error bars represent the standard error of the means (n = 5).

4. Discussion

Parasite-specific, non-host resistance to various pathogens has been frequently reported in the genus *Populus* comprising poplars, cottonwoods and aspens or white poplars [17–23]. Aspens and white poplars comprise the *Populus* section, and they were represented in this study by *P. tomentosa*. The *Populus* section is distinct from all other sections of the genus in terms of its pathogens [17], and it was expected that *P. tomentosa* would be a non-host for *M. larici-populina*. Prior to this study, studies have been of interspecific hybrids, typically *P. trichocarpa* × *P. deltoides*. One of the parental species, the allopatric one, was invariably not a host to a particular pathogen, but it maintained genes for resistance that segregated in the hybrids. Hybridization is possible and occurs naturally within sections of the genus and even between some sections such as *Tacamahaca* (e.g., *P. trichocarpa*) and *Aigeiros* (e.g., *P. deltoides*) [24,25]. All of the reports of parasite-specific, non-host resistance involved these intrasectional or intersectional hybrids of *Aigeiros* and *Tacamahaca*. However, hybridization is not possible between either of the latter two sections and the *Populus* section which comprises the aspens and white poplars. This is an important point in the context of the current study, since the *Populus* section includes *P. tomentosa*, the source of non-host resistance here.

The heteroecious life cycle of *M. larici-populina* must also be factored in to a discussion of new findings on the nature of non-host resistance to rust fungi. The fact that *M. larici-populina* also infects *Larix* spp. is germane because it superficially suggests that this rust is not so narrowly host-specific, at least at the level of the entire life cycle. However, each of the three infective spore states is narrowly host-specific. Aeciospores may be produced on *Larix* spp., but they can only infect susceptible hosts in *Populus*. Urediniospores are then produced on poplars, and each new uredinial generation can re-infect poplars. Basidiospores may be produced on fallen, overwintered poplar leaves, but they can only infect *Larix* spp. in spring.

The finding of this paper (i.e., a prehaustorial role for the cuticle in non-host resistance of *P. tomentosa* to *M. larici-populina*) is distinct in obvious ways from the previous examples of parasite-specific, non-host resistance [17–23]. Prehaustorial, cuticular resistance is not a trait that could segregate in hybrid progenies of *P. tomentosa* and a species in the *Aigeiros* or *Tacamahaca* sections since such hybrids are not possible. On the other hand, cuticular chemistry involves traits that could be parasite-nonspecific and thus an example of what Heath (1995) meant by non-host, or basic, resistance [26]. However, for the cuticle-based resistance of *P. tomentosa* to be clearly proven to be parasite-nonspecific, it would have to function not only against *M. larici-populina* but also against other poplar rusts. In fact, it would presumably function against all other rusts for which *P. tomentosa* is a non-host. An essential next step

would thus be to first test the cuticular extract and specific components of the *P. tomentosa* cuticle against such *Melampsora* species as *Melampsora medusae* that are also limited to the *Aigeiros* and *Tacamahaca* sections. *Melampsora medusae* has recently been reported from China [27].

Parasite-nonspecific resistance is theorized to account for non-host resistance via both inducible and constitutive mechanisms. The leaf cuticle could play a constitutive role; it is the first line of defense that a foliar pathogen encounters. The mechanism associated with our findings would seem likely to involve the chemical inhibition of spore germination and growth. By slowing the progress of *M. larici-populina* toward stomata, inhibition could provide effective resistance. Stomatal location and entry is, after all, a time-limited process. It is known that the clock for this process is effectively provided by the drying of the wet leaf surface [28]. Before the cuticle surface dries, the rust fungus must find a stomate, and the inhibitory cuticular compounds of *P. tomentosa* could sufficiently slow rust fungi to ensure failure. Our findings also allow us to predict that the inhibitory cuticular compounds reported here should fail to deter the one rust fungus, *M. magnusiana*, that does successfully parasitize *P. tomentosa* in China [27]. A corollary would be that the cuticle of *P. purdomii* should provide non-host resistance to *M. magnusiana*, and the cuticular compounds conferring resistance should differ from those identified here.

The cuticle is mainly comprised of cutin, and the latter is chemically similar to suberin [29]. Both form significant, multifunctional barriers [30]. Suberin is known to be involved in host resistance to pathogens as an inducible response [23]. Most importantly, the cuticle has clearly been a form of resistant barrier to rust fungi through evolutionary time. Many rust fungi cannot, in fact, penetrate the cuticle and have thus had to evolve as thigmotropic organisms able to locate stomata. Some of those that are capable of the direct penetration of the cuticle are like *M. larici-populina* in that their direct penetrations do not result in successful parasitism.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/10/870/s1, Figure S1: Symptoms of tomentosa, deltoides and purdomii poplar in the same field, respectively; Figure S2: GC-MS Chromatography of the three taxa poplars; Figure S3: Figure 3 Candidate cuticle role for uredinial density and germination; Table S1: Details of material samples; Table S2: Absolute and relative content of the candidate chemicals identified in three species poplar.

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