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Morphological and Biochemical Factors Associated with Constitutive Defense to Thrips in Alfalfa

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Abstract: Plants have evolved a series of inducible or constitutive defense mechanisms in response to herbivore attack. Constitutive plant defenses are morphological and biochemical traits of the plants themselves, regardless of the presence of herbivores. We bred an alfalfa variety (Caoyuan No.4) with high thrips resistance, but the mechanisms underlying Caoyuan No.4 resistance to thrips are not well understood. To explore the constitutive defense of Caoyuan No.4, the morphological and biochemical traits associated with constitutive defense to thrips in alfalfa were analyzed using a thrips-susceptible alfalfa accession (Caoyuan No.2) as a control. The results showed that Caoyuan No.4 had thicker palisade tissue and parenchyma tissue, wider collenchyma, phloem, cambium and lignin layer, and smaller epidermal cells and stomatal aperture compared to Caoyuan No.2. Moreover, Caoyuan No.4 showed more non-glandular trichomes in both leaves and stems, but less glandular trichomes and more wax in stems. In addition, the results of the widely targeted metabolomics analysis showed that metabolites related to flavonoid, isoflavonoid, flavone and flavonol biosynthesis, as well as cysteine and methionine metabolism, differed between CaoyuanNo.2 and Caoyuan No.4. These findings shed new light on the constitutive insect defense of plants associated with physical or biochemical traits and may provide convenient markers for breeding thrips-resistant alfalfa cultivars.

Keywords: *Medicago sativa*; thrips; constitutive defense; trichome; wax; metabolome



Citation: Zhang, Z.; Chen, Q.; Sa, R.; Dai, R.; Shuang, S.; Jiang, X.; Liu, H.; Tan, Y.; Tang, F.; Temuer, B.

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Agronomy **2022**, *12*, 1175. <https://doi.org/10.3390/agronomy12051175>

Academic Editor: Steven R. Larson

Received: 25 April 2022

Accepted: 12 May 2022

Published: 13 May 2022

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1. Introduction

Alfalfa (*Medicago sativa* L.) has become the most widely cultivated forage in China and is widely cultivated worldwide because of its high yield, high quality, and nitrogen fixation ability. However, insect pests seriously affect the yield and quality of alfalfa in the cultivation stage. To date, 297 kinds of alfalfa pests have been reported in China, belonging to 48 families in eight orders, and thrips have become one of the main insect pests affecting alfalfa production [1,2]. Thrips are tiny insects that are cell-content feeders, greatly affecting crops, ornamentals and forages worldwide. Thrips can cause both direct feeding damage by consuming sap from phloem tissue and indirect damage by vectoring tospoviruses, resulting in decreased forage yield and quality [3,4]. Thrips are one of the common pests on alfalfa, mainly including *Odontothrips loti*, *Thrips vulgatissimus*, *Thrips major*, *Haplothrips aculeatus* as many as a dozen [5]. Moreover, *Odontothrips loti* is the dominant thrips species of alfalfa in China, which can cause as high as 70% to 100% plant damage during the

entire growth period of alfalfa [6–8]. It has been reported that thrips cause 10–30% grass yield loss every year in China [9]. However, thrips control is a challenge because of their highly polyphagous and complex lifestyle [10]. Currently, management and control of thrips mainly relies on chemical pesticides, which are likely to induce thrips resistance to insecticides, ecosystem damage and food safety issues [10,11]. For instance, pesticides such as imidacloprid, acetamiprid, emamectin and spinosad all showed good control effect on *Odontothrips loti* in alfalfa fields [12]. Biological control of *Odontothrips loti* with anthocorid predators, *Orius minutus* and *Orius niger* showed good control effect [13]. Moreover, integrated pest management (IPM) guidelines for crops emphasize the use of a range of tactics to reduce thrips abundance and damage, rather than reliance on insecticides [14,15]. Thus, breeding thrips-resistant crops and forages is an important alternative strategy to decrease losses and damage caused by thrips [16,17]. Plants have evolved a suite of defense mechanisms to ward off herbivorous pests, including constitutive defense and inducible defense mechanisms [18,19]. Recent investigations have focused on thrips-induced plant defenses [20–22] as well as constitutive plant defenses [17,23]. Constitutive plant defenses include physical or morphological and biochemical defenses. Usually, morphological traits, such as glandular hairs, trichomes, waxy cuticles, silicon and cell walls, are the first line of defense to deter insects from feeding [23–25], while biochemical defenses, including the production of toxins or metabolites, regulate the second line of defenses [23,26]. Some recent studies have investigated aspects of the relationship between alfalfa and thrips, including the mechanisms of plant defense [26,27], and the morphology and distribution of thrips [28].

Since there is an increasing focus on improving crop production through safe and sustainable means by reducing the reliance on pesticides [29,30], the use of resistant cultivars is currently considered the most effective and environmentally sustainable strategy to control insects. Alfalfa cultivars are heterogeneous populations, and recurrent selection is highly heritable, and it is relatively easy to accumulate excellent genes through a large population. In our previous study, an alfalfa variety (Caoyuan No.4) with high thrips resistance (hazard point coefficient: 0.26, and pest index: 0.33) was bred through nearly 30 years of consecutive field recurrent selection [7,31]. However, the mechanisms underlying Caoyuan No.4 resistance to thrips are not well understood. Thus, to better understand the mechanisms of constitutive thrips resistance in Caoyuan No.4, morphological and biochemical traits were analyzed using the thrips-resistant alfalfa accession (Caoyuan No.4) and compared with a thrips-susceptible alfalfa accession (Caoyuan No.2).

2. Materials and Methods

2.1. Plant Growth and Treatments

Two alfalfa cultivars, Caoyuan No.4 (a thrips-resistant alfalfa cultivar) and Caoyuan No.2 (a thrips-susceptible cultivar) [7], were cultivated in pots (H 21 cm × D 14 cm, one plant per pot) containing field collected soil in a greenhouse with a relative humidity of $60 \pm 5\%$ and $70 \pm 5\%$, at $30 \pm 5\text{ }^\circ\text{C}$ and $20 \pm 5\text{ }^\circ\text{C}$, during day and night, respectively. Both cultivars were bred at Inner Mongolia Agricultural University, China [21]. The variety Caoyuan No.4 was bred through recurrent phenotypic selection among a base population created by more than 400 different alfalfa cultivars, while the variety Caoyuan No.2 was bred through multiple parental pollination, with one female parent and five mixed male parents. In addition, both the varieties are drought and cold resistant cultivars, but Caoyuan No.4 is a thrips-resistant alfalfa cultivar [7].

When the alfalfa seedling reached budding stage (about 60 days), the top 3–4 leaves or the corresponding part of stems were collected from each cultivar for subsequent analysis, with three biological replicates.

2.2. Thrips Resistance Evaluation

Alfalfa plants were treated as described by Zhang et al. [21] and Tu et al. [4] with some modifications. When the alfalfa seedlings had reached budding stage (about 60 days), about

50 alfalfa plants of each cultivar were randomly selected and arranged and covered by a big cage with 90-mesh nylon. A number of *Odontothrips loti* (30 per branch) was inoculated onto the leaves of each plant. Seven days later, 10 of the thrips feeding on alfalfa plants of each cultivar were randomly selected for thrips resistance evaluation with five replicates.

The thrips resistance of alfalfa was evaluated by insect index and was performed as in our previous study [7]. Insect resistance identification was carried out according to the insect index. Insect index is a comprehensive index that comprehensively considers both insect prevalence rate and severity (Table 1). The insect index of a highly resistant cultivar is usually less than 0.5. Taking leaves as the unit, it was calculated with the following formula:

$$\text{Insect index} = \sum \frac{X_n}{n} \sum X = \frac{X_{0a} + X_{1a_1} + \dots + X_{na_n}}{nT}$$

$a_0, a_1 \dots a_n$, severity level;

n , highest severity level;

T , total plant number;

Table 1. Severity levels.

Severity Level	Severity Level (%)
0	0
1	0~4
2	5~9
3	10~19
4	20~29
5	30~49
6	50~100

2.3. Morphological Measurements

Morphological resistance traits were measured for the top 3–4 leaves or the corresponding part of stems of each replicate. The blade structure of leaves was identified in sections using histochemistry and microscopy. Briefly, leaf discs were fixed in FAA (50 mL of 40% formaldehyde, 50 mL of glacial acetic acid, and 90 mL of 50% ethanol) for 24 h, dehydrated with a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95%, 100%), after which ethanol was exchanged with acetone solution, and then embedded in paraffin. Sections of 5 mm thickness were cut using a Leica Ultracut R (LEICA, Frankfurt, Germany). Photographs were taken using a Motic BA210 stereo microscope system with a Motic Images Plus 2.0 M. In addition, leaf surface scanning electron photomicrographs were taken as described by Jia et al. [32]. Blades and stems were vacuum fixed in 3% glutaraldehyde at 4°C overnight and then washed 3–5 times with fresh PBS solution. The samples were fixed in 1% osmic acid for 2 h, washed with fresh PBS solution (Sodium dihydrogen phosphate 38.0 g, and disodium hydrogen phosphate 5.04 g, add water to make 1000 mL) and then dehydrated in an ethanol series and isoamyl acetate. The dried samples were installed on aluminum stubs and then coated with gold palladium using an ion sputter coater (ISC 150, SuPro, Shenzhen, China). Trichome (glandular hair and non-glandular hair) and cuticular wax were examined under a Hitachi SU-8010 Scanning Electron Microscope (HITACHI, Tokyo, Japan).

The top 3–4 leaves were collected from each plant of each cultivar. Wax metabolites were extracted with chloroform as described by Mirka [33]. After evaporation of the chloroform, wax content was determined by using a gas phase hydrogen flame detector (GC-FID) [33].

The widely targeted metabolomics analysis of leaves of both cultivars was performed using methods described in our previous study [21]. The sample processing, extraction, and metabolites detection were performed by Biomarker Technologies Co., Ltd. (Beijing, China) following their standard procedures [34]. Quadrature signal correction partial least

squares-discriminant analysis (OPLS-DA) and variable importance in projection (VIP) were used to obtain the maximum differences between the cultivars. Metabolites with $VIP > 1.0$, fold change ≥ 2 and fold change ≤ 0.5 were considered to be differential metabolites for group discrimination. The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used to annotate and display the differential metabolites [35].

3. Results

3.1. Thrips Resistance Identification of Caoyuan No.4 and Caoyuan No.2

Cultivating resistant cultivars with both improved agronomic traits and significant thrips resistance could be an alternative to insecticidal control of thrips. Screening to identify thrips-resistant accessions and revealing the thrips-resistance mechanism are very important in this approach. Some reports are available where cultivated germplasm was screened for breeding thrips resistant alfalfa [4,6,7,29]. In this study, thrips resistance identification was carried out according to the insect index. As shown in Figure 1, the insect index of Caoyuan No.2 was significantly higher than that of Caoyuan No.4 ($p \leq 0.05$). The insect indexes of Caoyuan No.2 and Caoyuan No.4 were 0.81 and 0.45, respectively. We generally consider that the insect index of a highly resistant cultivar would be less than 0.5, while the insect index of a sensitive cultivar is often greater than 0.5 [6]. These results confirmed that Caoyuan No.4 is a thrips resistant cultivar, while Caoyuan No.2 is a thrips sensitive cultivar. Thus, both cultivars were suitable for the further study of constitutive defense to thrips in alfalfa.

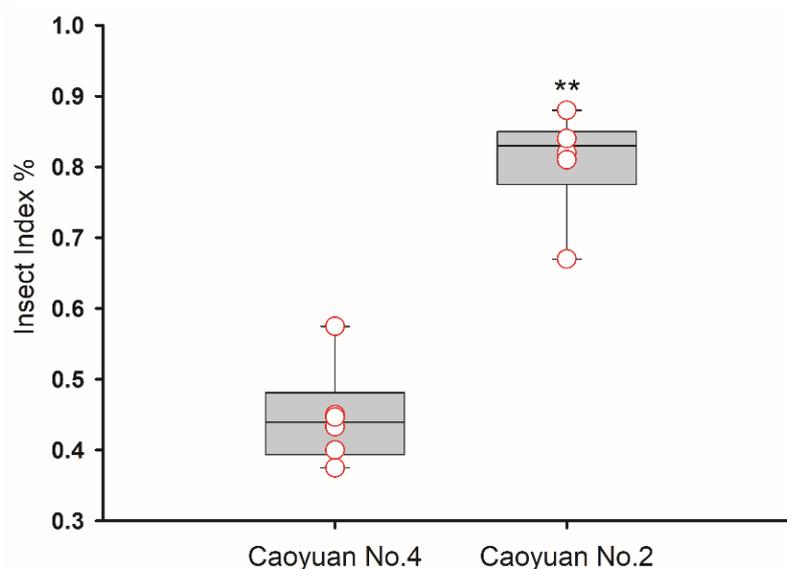


Figure 1. Insect index of Caoyuan No.2 and Caoyuan No.4. Bars represent the mean \pm SE ($n = 3$), double asterisk indicates $p \leq 0.01$.

3.2. Morphological Factors

Thrips resistance in many plants has been attributed to plant morphological characteristics such as leaf position [36], toughness or structure [37,38], stomatal characteristics [39], trichomes (glandular hair and non-glandular hair) [40–43] and epicuticular wax [23,33,44], which could restrict insect attack or oviposition. Many studies have been correlative and there is little consensus concerning the role of epidermal wax and trichomes in thrips defense. For example, increased leaf wax was associated with resistance against thrips in cabbage [45], while cultivars with less wax provided more protection against thrips than those with more wax in onion [46]. It is apparent from Figure 2 that the leaf structure of Caoyuan No.2 and Caoyuan No.4 were different. The collenchyma and phloem width, cambium and the lignin layer thickness of leaves (main vein) in Caoyuan No.4 were larger than those of Caoyuan No.2. Compared with Caoyuan No.2, the thickness of leaf palisade

tissue in Caoyuan No.4 was also larger. In addition, the leaves of Caoyuan No.2 showed long club-shaped palisade cells with big air-chambers under the epidermal cells (Figure 2), and Caoyuan No.4 had smaller epidermal cells which were more densely packed than in Caoyuan No.2 (Figure 3A,B). These results were similar to those of Dinar et al. [23], who also found that the resistant varieties showed shorter mesophylls and thicker palisade tissue and parenchyma tissue.

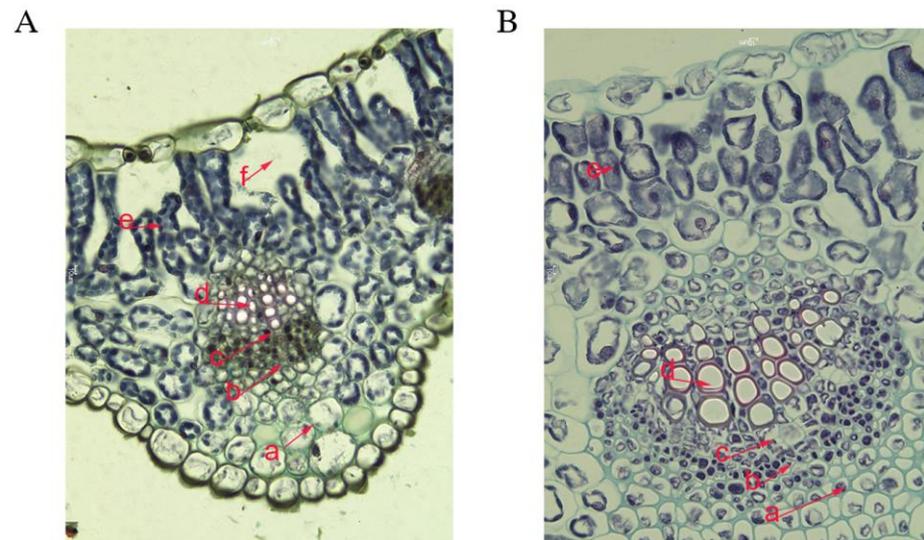


Figure 2. Structural comparison of Caoyuan No.2 and Caoyuan No.4. (A) Leaf cross section of Caoyuan No.2. (B) Leaf cross section of Caoyuan No.4. a, collenchyma; b, phloem; c, cambium; d, lignin layer; e, palisade tissue; f, air-chamber.

An ultrastructural examination of leaves found that Caoyuan No.4 showed fewer and smaller stomata than Caoyuan No.2, and the stomatal aperture of Caoyuan No.4 was also smaller than that of Caoyuan No.2 (Figure 3A,B). These findings are consistent with those of other investigators who observed that resistant cultivars showed the highest but smallest stomata than sensitive cultivars [39].

The cuticular wax layer of plants is a physical barrier for the feeding or oviposition of insects on the plant surface [47]. Several studies have documented that cuticular wax or wax metabolites can be used for host plant recognition and as feeding stimulants by insects, such as flea beetles, sawflies and thrips [33,48,49]. As shown in Figure 3A–D, the results of leaf surface scanning electron photomicrographs showed that there was more cuticular wax of stems in Caoyuan No.4 than Caoyuan No.2, while there was no significant difference in cuticular wax on leaves between the two cultivars. In accordance with these results, the wax content of stems determined by a gas phase hydrogen flame detector in Caoyuan No.4 was 2.48-fold that of Caoyuan No.2, while the wax content of leaves between the cultivars was not significantly different ($p \leq 0.05$) (Figure 4). In accordance with our results, previous studies have demonstrated that stalk surface wax components contribute towards resistance to insects in sugarcane [50].

The role of trichomes in thrips defense is also controversial in different plant species. Soybean genotypes with low densities of trichomes showed reduced thrips damage [37], while cultivars with high trichomes displayed lower thrips infestations in strawberry [51]. Moreover, experiments conducted with tomato cultivars suggested that its resistance to western flower thrips was independent from trichome [42]. In this study, we found that both the leaves and stems of Caoyuan No.4 showed higher densities of non-glandular trichome than those of Caoyuan No.2 (Figure 3E–H). In particular, the non-glandular trichomes of leaves in Caoyuan No.4 were shorter and more erect than those of Caoyuan No.2 (Figure 3E,F). However, the stems of Caoyuan No.2 showed more glandular trichome than Caoyuan No.4 (Figure 3G,H). These results suggested that both glandular and non-

glandular trichome may play important roles in constitutive defense to thrips in alfalfa. The short, erect and high density non-glandular trichome in Caoyuan No.4 might prevent thrips from feeding or oviposition. However, we suggest that the negative correlation of glandular trichome in stems with thrips resistance in alfalfa might be due to derived volatile compounds, such as sucrose and malonylated flavone glycosides, which have been related to susceptibility [52]. In summary, these results clearly indicated that morphological factors, including leaf structure, cuticular wax of stems, and trichomes may all be involved constitutive defense to thrips in alfalfa.

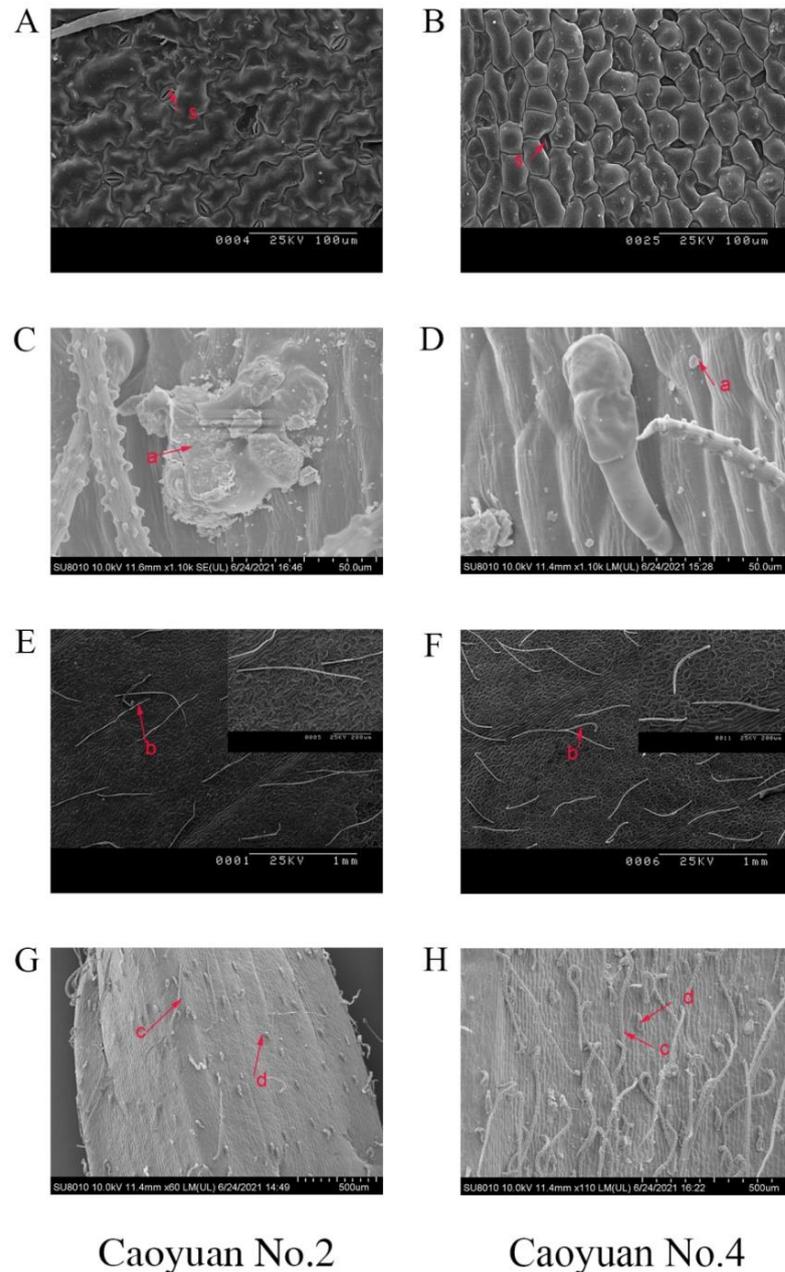


Figure 3. Leaf and stem surface scanning electron photomicrographs of Caoyuan No.2 and Caoyuan No.4. (A) Epidermal cells and stomatal features of Caoyuan No. 2. (B) Epidermal cells and stomatal features of Caoyuan No.4. (C) Epidermal wax of stem in Caoyuan No. 2. (D) Epidermal wax of stem in Caoyuan No.4. (E) Trichomes of leaf in Caoyuan No. 2. (F) Trichomes of leaf in Caoyuan No. 4. (G) Trichomes of stem in Caoyuan No. 2. (H) Trichomes of stem in Caoyuan No. 4. a, wax; b, non-glandular trichomes; c, non-glandular trichomes; d, glandular trichomes; s, stomata.

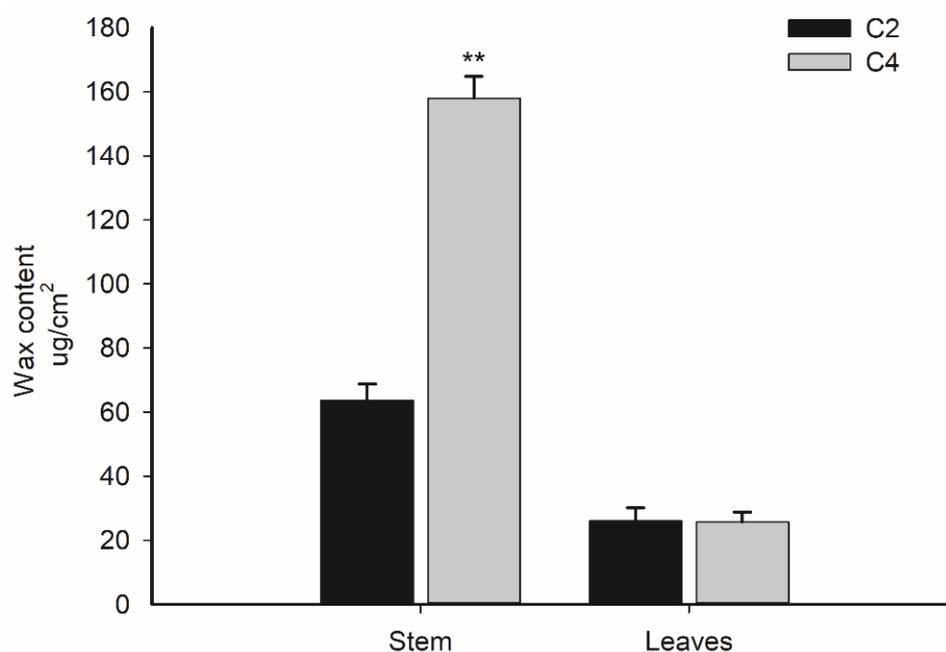


Figure 4. Wax content of Caoyuan No.2 and Caoyuan No.4 determined using a gas phase hydrogen flame detector. C2, Caoyuan No.2; C4, Caoyuan No.4. Bars represent the mean \pm SE ($n = 3$), double asterisks indicate $p \leq 0.01$.

3.3. Biochemical Factors

Plants have evolved both direct and indirect defense mechanisms to herbivores [18]. Both direct and indirect defenses can be displayed through constitutive plant defense mechanisms. Several reports have shown that plant biochemical factors, including primary and secondary metabolites, play important roles in both constitutive and induced defense to herbivores [53–55]. It is known that widely targeted metabolomics analysis provides an effective qualitative and quantitative method to determine the metabolites in plant responses to abiotic or biotic stress [21,56,57]. In this study, widely targeted metabolome was used to analyze the metabolites related to constitutive thrips defense in alfalfa. As shown in Tables 2 and S1, a total of 776 metabolites were divided into 23 classes, including 102 organic acids and derivatives, 97 flavones, 93 amino acid and derivatives, 72 lipids, 64 phenylpropanoids, 54 nucleotide and derivatives, 36 alkaloids, 30 flavonols, 27 flavonoid, 26 phenolamides, 26 flavanones, 23 terpene, 21 vitamins and derivatives, 19 alcohols, 17 carbohydrates, 14 isoflavone, 11 anthocyanins, six polyphenol, six indole derivatives, five sterides, three quinones, one proanthocyanidins and 31 others (Table S1).

Moreover, there were 28 and 14 metabolites belonging to 14 and seven classes determined either in Caoyuan No.2 or in Caoyuan No.4, respectively (Figure 5A, Table S2). These results suggest that Caoyuan No.2 and Caoyuan No.4 have different metabolic profiles. What stands out here is that several different phenolamides metabolites were detected in either Caoyuan No.2 or Caoyuan No.4. In particular, N-Caffeoyl agmatine, N-p-Coumaroyl agmatine, N-hexosyl-p-coumaroyl putrescine, N-(4'-O-glycosyl)-p-coumaroyl agmatine and N-Feruloyl agmatine were only detected in Caoyuan No.2, while N', N''-di-p-coumaroylspermine, N-p-coumaroylspermine, N'-p-coumaroylspermine, N-p-Coumaroyl spermidine and N-Acetyl tryptamine were only detected in Caoyuan No.4 (Table S2). This finding is consistent with that of other investigators who found that a different diversity of phenolamides showed exactly the opposite effect in plant defense to thrips [58,59]. Furthermore, four phenylpropanoids, including resveratrol, phenethyl caffeate, isoacteoside and O-feruloyl coumarin were only detected in Caoyuan No.2, suggesting that these phenylpropanoids might be negatively correlated with thrips constitutive defense in plants.

Table 2. Overview of annotated metabolites in Caoyuan No.2 and Caoyuan No.4.

Type	Number	Percentage
Organic acids and derivatives	102	13.01%
Flavone	97	12.37%
Amino acid and derivatives	93	11.86%
Lipids	72	9.18%
Phenylpropanoids	64	8.16%
Nucleotide and derivatives	54	6.89%
Alkaloids	36	4.59%
Flavonol	30	3.83%
Flavonoid	27	3.44%
Phenolamides	26	3.32%
Flavanone	26	3.32%
Terpene	23	2.93%
Vitamins and derivatives	21	2.68%
Alcohols	19	2.42%
Carbohydrates	17	2.17%
Isoflavone	14	1.79%
Anthocyanins	11	1.40%
Polyphenol	6	0.77%
Indole derivatives	6	0.77%
Sterides	5	0.64%
Quinones	3	0.38%
Proanthocyanidins	1	0.13%
Others	31	3.95%
Total	776	100%

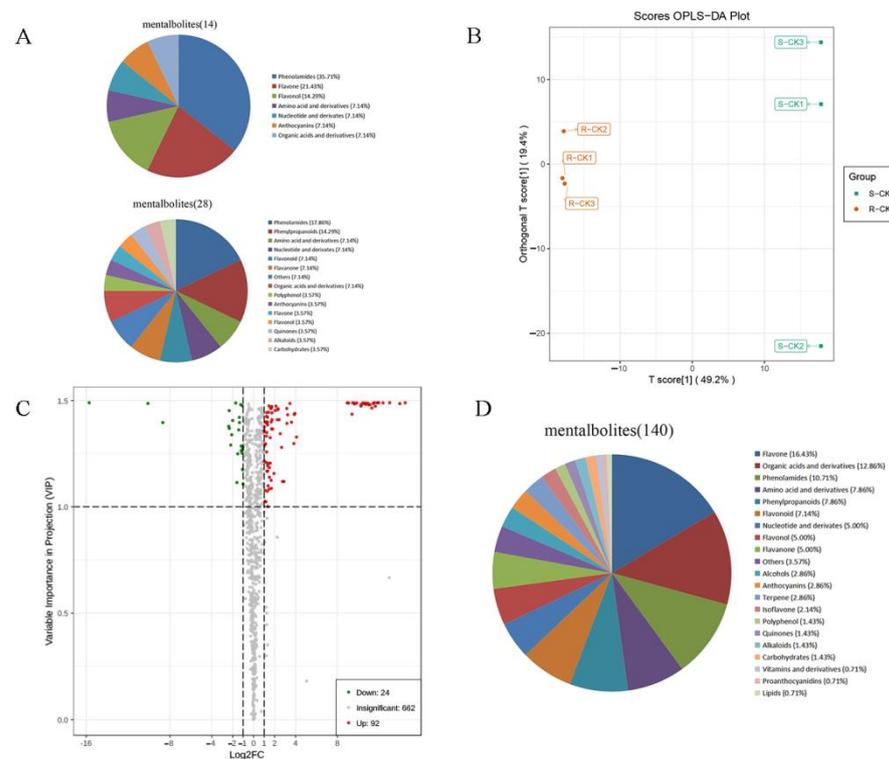


Figure 5. Differentially accumulating metabolites between Caoyuan No.2 and Caoyuan No.4. (A) Differentially accumulating metabolites determined in either Caoyuan No. 2 or Caoyuan No. 4. (B) The score plots of orthogonal partial least-squares discriminant analysis (OPLS-DA); S-CK, Caoyuan No.2; R-CK, Caoyuan No.4. (C) Upregulated and downregulated metabolites detected in Caoyuan No.2 compared with Caoyuan No.4 (D) Pie chart depicting the biochemical categories of the differential metabolites identified between Caoyuan No.2 and Caoyuan No.4.

Orthogonal partial least-squares discriminant analysis (OPLS-DA) is a multivariate statistical analysis method with supervised pattern recognition that can effectively eliminate factors that are not related and can be used to screen differential metabolites [60]. The score plots from OPLS-DA showed that in the two cultivars, the chestnuts with different thrips-resistance were separated, indicating that the metabolic differences were significant (Figure 5B).

The fold change value and VIP value were combined to screen the differential accumulated metabolites (DAMs). As described previously [21], metabolites with $|\log_2FC| \geq 1$ and $VIP \geq 1$ were considered as DAMs for group discrimination. As shown in Figure 5C, a total of 92 upregulated and 34 downregulated metabolites were detected in Caoyuan No.2 compared with Caoyuan No.4 ($VIP > 1$ and $|\log_2FC| \geq 1$). The 140 DAMs were categorized into 19 different classes, but the majority were phenolamides, phenylpropanoids, amino acid and derivatives, organic acids and derivatives, flavonoids, flavone and flavonol (Figure 5D, Table S3). These findings would seem to suggest that both primary metabolites, such as amino acid and organic acids, and secondary metabolites, including flavonoids, alkaloids and terpenoids, may be involved in thrips constitutive defense in alfalfa. In accordance with our results, previous studies have demonstrated that the majority of specialized metabolites were involved in constitutive defense against thrips in various plants [61–63].

Differential accumulated metabolites interact in organisms to form different pathways. According to the KEGG annotation and enrichment results, the relative metabolic pathways are shown in Figure 6. It was shown that the DAMs were most significantly enriched to flavonoid biosynthesis (ko00941), isoflavonoid biosynthesis (ko00943), flavone and flavonol biosynthesis (ko00944), and cysteine and methionine metabolism (ko00270). Interestingly, this accorded with our previous observations on thrips-induced defense in alfalfa [19]. These results suggested that the pathways, such as flavonoid biosynthesis (ko00941), isoflavonoid biosynthesis (ko00943) and flavone and flavonol biosynthesis (ko00944), contributed to both constitutive and thrips-inducible plant defense in alfalfa. The roles of flavonoids or isoflavonoids in plants thrips defense have also been reported by other investigators [4,17,64].

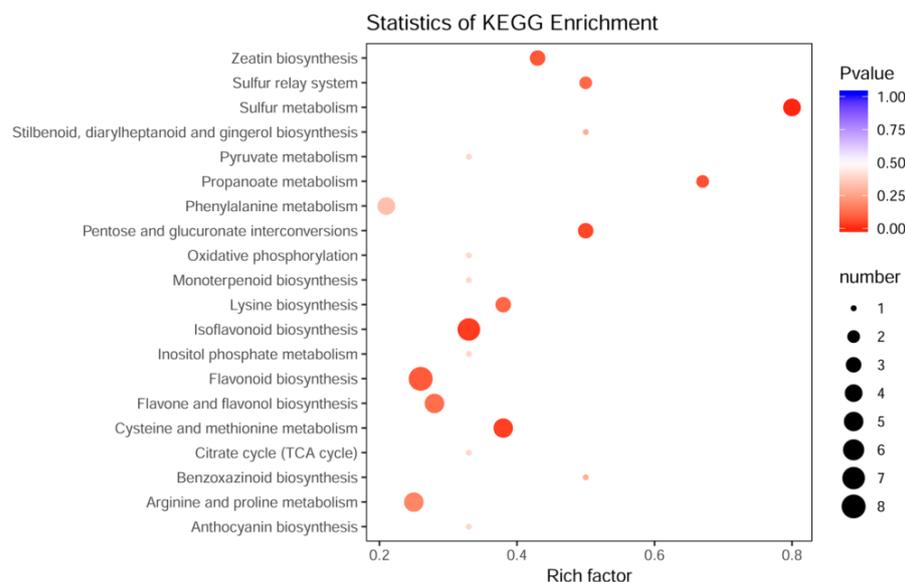


Figure 6. Scatter plot of KEGG pathways of the differentially accumulated metabolites enriched. The degree of enrichment is shown by Rich factor p -value, and the number of metabolites enriched in each pathway.

In particular, the DAMs of Caoyuan No.4 in cysteine and methionine metabolism, including 5'-Deoxy-5'-(methylthio) adenosine (pme1473), DL-Homocysteine (pme0057), L-Homoserine (pme0161), L-Cysteine (pme0195) and S-(5'-Adenosyl)-L-methionine (pme2735),

were all significantly higher than those in Caoyuan No.2, increasing in the former by up to 3.29, 3.74, 2.22, 3.73 and 2.55-fold compared to Caoyuan No. 2, respectively (Figures 7A and S1). This indicated that metabolites related to cysteine and methionine metabolism may be positively correlated with thrips defense in alfalfa. By contrast, the DAMs of Caoyuan No.4 involved in isoflavonoid biosynthesis, including vestitol (pmf0113), maackiain (pmf0112), biochanin A (pme3250), coumestrol (pmf0117), medicarpin (pmf3245), sissotrin (pmf3400) and prunetin (pmf3292), were all significantly lower than those in Caoyuan No.2, decreasing to 0.26, 0, 0, 0.06, 0.37 and 0.31-fold of Caoyuan No.2, respectively (Figures 7B and S2). This indicated that these metabolites related to isoflavonoid biosynthesis may be positively correlated with thrips defense in plants. For flavonoid biosynthesis, the contents of different DAMs behaved differently in the two cultivars. DAMs including dihydromyricetin (pme2898), naringin (pme0331), garbanzol (pmf0108) and tricetin (pme3303) in Caoyuan No.2 were significantly higher than those in Caoyuan No.4, while phloridzin (pmf0583), kaempferol (pme0200), hesperetin 7-O-neohesperidoside (pme0001) and epigallocatechin (pme1514) were significantly lower or absent (Figures 7C and S3). Except for kaempferol (pme0200), the content of DAMs (such as kaempferide, rhoifolin, apiin and cosmosiin) involved in flavone and flavonol biosynthesis in Caoyuan No.4 were significantly higher than those in Caoyuan No.2 (Figures 7D and S4). In accordance with our results, the majority of previous studies have demonstrated that metabolites including flavonoid and other secondary metabolites contributed to thrips resistance, either in the presence or absence of morphological traits [65–67]. However, the correlative roles of most compounds in thrips resistance have not been functionally characterized and identified.

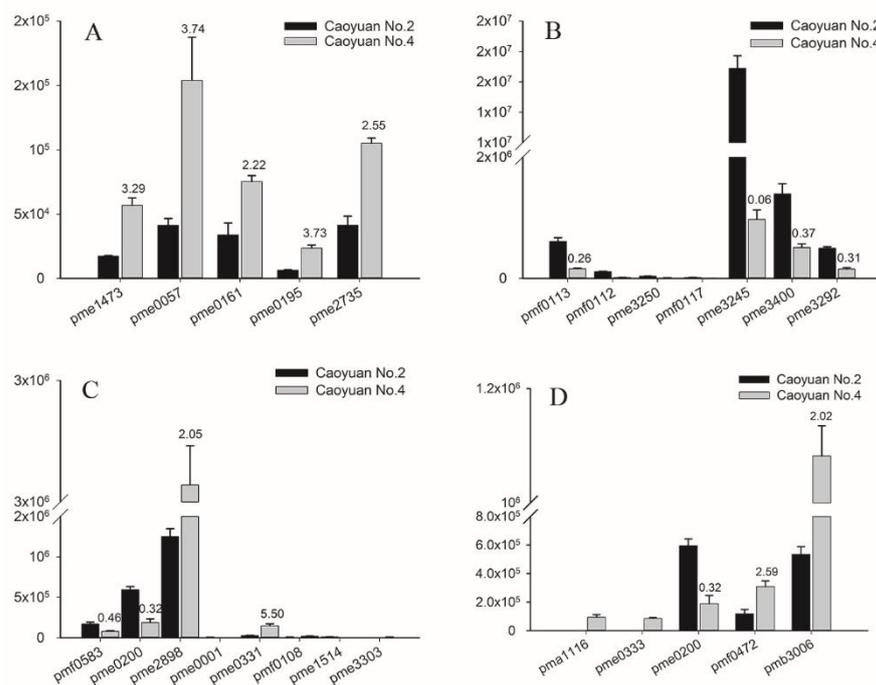


Figure 7. The relative content of differential accumulated metabolites between Caoyuan No.2 and Caoyuan No.4 in KEGG enrichment pathways. (A) The relative content of differential accumulated metabolites in cysteine and methionine metabolism (ko00270); (B) The relative content of differential accumulated metabolites in isoflavonoid biosynthesis (ko00943); (C) The relative content of differential accumulated metabolites in flavonoid biosynthesis (ko00941); (D) The relative content of differential accumulated metabolites in flavone and flavonol biosynthesis biosynthesis (ko00941).

Together, these results provide important insights into an association between biochemical factors, including primary and secondary metabolites and constitutive thrips defense in alfalfa. Biochemical factors involved in flavonoid, isoflavonoid, flavone and

flavonol biosynthesis, as well as cysteine and methionine metabolism, may play important roles in constitutive thrips defense in alfalfa.

4. Conclusions

The present study was designed to determine the morphological and biochemical factors associated with constitutive defense to thrips in alfalfa. Our findings clearly indicate that both morphological and biochemical factors contributed to constitutive defense to thrips in alfalfa. Among morphological factors, we found that the leaf structure including the thickness of palisade tissue and parenchyma tissue, the width of collenchyma, phloem, cambium and the lignin layer, the size of epidermal cells, stomatal aperture, and the densities and characteristics of glandular and non-glandular trichomes, as well as stem surface wax components, were all involved in constitutive defense to thrips in alfalfa. The second major finding was that metabolites related to flavonoid, isoflavonoid, flavone and flavonol biosynthesis, as well as cysteine and methionine metabolism, may be the most important biochemical factors in constitutive thrips defense in alfalfa. These findings have shed new light on constitutive insect defense of plants through physical or biochemical traits. We further suggest that the morphological traits of plants, such as wax, trichomes and leaf structure, may provide convenient markers for breeding thrips-resistant alfalfa cultivars. However, the correlative roles of the morphological and biochemical traits in thrips resistance should be further functionally characterized by conducting bioassays with thrips.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12051175/s1>, Table S1: Details of metabolites detected in Caoyuan NO.2 and Caoyuan No.4. Table S2: Metabolites determined either in Caoyuan No.2 or Caoyuan No.4. Table S3: Details of differentially accumulating metabolites between Caoyuan No.2 and Caoyuan No.4. Figure S1: KEGG pathway diagram of differential accumulated metabolites between Caoyuan No.2 and Caoyuan No.4 mapped to the cysteine and methionine metabolism. Figure S2: KEGG pathway diagram of differential accumulated metabolites between Caoyuan No.2 and Caoyuan No.4 mapped to isoflavonoid biosynthesis. Figure S3: KEGG pathway diagram of differential accumulated metabolites between Caoyuan No.2 and Caoyuan No.4 mapped to the flavonoid biosynthesis. Figure S4: KEGG pathway diagram of differential accumulated metabolites between Caoyuan No.2 and Caoyuan No.4 mapped to the flavone and flavonol biosynthesis.

Author Contributions: Conceptualization, Z.Z.; Data curation, Q.C. and R.D.; Funding acquisition, Z.Z. and B.T.; Investigation, R.S., X.J. and H.L.; Methodology, S.S.; Resources, Y.T. and F.T.; Writing—review and editing, Z.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by projects of the National Natural Science Foundation of China (32160333, 32060388), the National Natural Science Foundation of Inner Mongolia, China (2021MS03011) and Key Projects in Science and Technology of Inner Mongolia, China (2021ZD0031).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article and Supplementary Material.

Acknowledgments: We thank Guodong Han and Yunbo Wang from Inner Mongolia University for helpful comments and suggestions and Andreas Wilkes for English language editing.

Conflicts of Interest: The authors declare no conflict of interest.

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