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Chemometric analysis of the secondary metabolite profile of Yarrow (*Achillea collina* Becker ex Rchb.) affected by phloem feeding *Myzus persicae* Sulzer aphids

Abstract - Yarrow (*Achillea collina* Becker ex Rchb.) has a high content of secondary metabolites including phenolic acids. Among them, hydroxycinnamic acid such as chlorogenic acid and its derivatives were found to be the most abundant ones. The phloem feeding *Myzus persicae* Sulzer was hypothesized to affect the contents of secondary metabolites and change the metabolite profile. A high-performance liquid chromatography technique (HPLC) was used to evaluate whether there is a difference in the phenolic profile between aphid infested and non-infested yarrow leaves. *M. persicae* colonies composed of between 20 and 30 individuals were allowed to feed for 10 and 20 days. Preprocessing was carried out to standardize the procedures in order to obtain optimal separation of analytes, good chromatographic peak shape and robustness of the results. The methanol extracts of leaves were analyzed by means of HPLC, and the time series of peak areas obtained from each extract were evaluated through chemometric analyses. Results of the phenolic fingerprints showed a specific chromatographic profile with 58 peaks. An autoregression analysis demonstrated the absence of correlation. The discriminant analysis carried out with the data satisfying the assumption of the absence of collinearity showed a significant effect of phloem feeding on soluble phenolic compounds and identified two peaks that separate aphid infested from non-infested plants. The hydroxycinnamic acids widely found in *A. collina* leaves were not affected by *M. persicae* feeding. The results are the basis for the current studies aiming at the identification of chemical compounds that correspond to the peaks.

Riassunto - *Analisi chemometrica del profilo dei metaboliti secondari di* Achillea collina *Becker ex Rchb. infestata da afidi appartenenti alla specie* Myzus persicae *Sulzer*

Achillea collina Becker ex Rchb. è una pianta medicinale caratterizzata da un alto contenuto in metaboliti secondari, in particolare in acidi fenolici. Tra questi, gli acidi idrossicinnamici quali il clorogenico e suoi derivati sono risultati essere i più abbondanti. Variazioni qualitative e quantitative di tali metaboliti sono state osservate in seguito ad attacchi entomatici. Si ipotizza che *Myzus persicae* Sulzer, afide che si nutre di sostanze floematiche, possa modificare il contenuto degli acidi fenolici della pianta. E' stata impiegata la tecnica di cromatografia liquida ad alta

prestazione (HPLC) per valutare se vi sono differenze nel profilo degli acidi fenolici in estratti metanolici di foglie di *A. collina* non infestate e infestate con *M. persicae*. Ad una colonia di afidi costituita da 20-30 individui è stato permesso di nutrirsi sulle piante per 10 o 20 giorni. Una procedura di messa a punto del metodo analitico ("preprocessing") è stata effettuata per standardizzare l'analisi, così da ottenere una ottimale separazione e risoluzione degli analiti, garantendo la robustezza dei risultati. Le sequenze delle aree dei picchi ottenute da ciascun estratto sono state valutate con procedure chemometriche. Il profilo dell'estratto metanolico caratteristico è risultato avere 58 picchi cromatografici. Analisi di auto regressione dimostrarono l'assenza di correlazione tra i picchi. L'analisi discriminante effettuata sui dati che soddisfano l'ipotesi di assenza di collinearità ha mostrato un effetto significativo della presenza degli afidi sui composti fenolici presenti nell'estratto metanolico. Due picchi, in particolare, sono risultati essere discriminanti tra piante non infestate e piante infestate. Gli acidi idrossicinnamici precedentemente identificati in foglie di *A. collina* non sono stati influenzati dall'attacco di *M. persicae*. I risultati fin qui ottenuti hanno posto la base per gli attuali studi, che hanno lo scopo di identificare la struttura chimica delle sostanze corrispondenti ai due picchi discriminanti.

Key words: *Achillea collina* Becker ex Rchb, *Myzus persicae* Sulzer, secondary metabolites, hydroxycinnamic acids, chemometrics, autoregression, discriminant analysis.

INTRODUCTION

Secondary metabolites, i.e. organic compounds that are not directly involved in the normal growth, development, or reproduction of organisms (Fraenkel, 1959; Dixon, 2001; Wink, 2003; Benderoth *et al*., 2006), are widely distributed in plants (Ramachandra Rao *et al*., 2002). In response to biotic and abiotic stresses, plants may increase the production of these substances and select different pathways for their synthesis (Grace & Logan, 2000; Hadacek, 2002; Wink, 2003). A complex genetic system contributes to the shaping of single and multiple signaling pathways and to the rapid accumulation of secondary metabolites which play an important role during disease infection or herbivore attack (Hartmann, 2004; Edreva *et al*., 2008, Walling, 2000). The current literature emphasizes the role of the secondary metabolites in plant herbivore interaction, although the plant is known to react with other biochemicals and biophysical elements to herbivore attack (Schowalter, 2006; Wittstock *et al*., 2004). The accumulation of phenolic compounds, emission of a higher number of volatiles, generation of hydrogen peroxide, localized cell death and synthesis of hydrolytic enzymes, often accompany the symptoms of stenophagous herbivore and pathogen attack (Moran *et al*., 2002). Because of their economic importance, phloem feeding aphids have received particular attention in investigations on secondary metabolites (Pichersky *et al*., 2000; Moran *et al*., 2002). Aphids have the potential to rapidly increase their population densities and phloem sap consumption (Kuśnierczyk *et al*., 2008).

Yarrow, *Achillea collina* Becker ex Rchb. belonging to the Asteraceae family, has a high content of secondary metabolites of interest in human medicine. Namely, aqueous

and alcoholic extracts have digestive, antiphlogistic, spasmolytic, stomachic, carminative, estrogenic properties (Benedek et al., 2007). Previous investigations pointed out that the SPAK cultivar is rich in hydroxycinnamic acids such as chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid (Giorgi *et al*., 2009). These compounds are produced in the shikimate pathway and implicated in structural support, pigmentation and mechanisms that increase the plant capacity to react to stress by scavenging reactive oxygen species (Bennett & Wallsgrove, 1994; Dixon & Paiva, 1995; Wink, 2003). Recent work reported that oils distilled from several species of *Artemisia* and *Achillea* showed some promise as aphid repellents (Halbert *et al*., 2008). Yarrow is a host plant for several aphids including the green peach aphid *Myzus persicae* Sulzer, which is a phloem feeding insect infesting plants belonging to more than 30 plant families (van Enden *et al*., 1969, Hill, 1983). *M. persicae* is widely used in ecological studies due to the versatility in colony maintaining (Moran *et al*., 2002).

The purpose of this work is to evaluate the response of yarrow to phloem feeding by *M. persicae* on the basis of phenolics. Specifically, the study evaluates whether there is a difference in phenolic metabolites between aphid infested and non-infested plants and aims to identify the compounds separating infested from non-infested plants. This is done by relying on the High Performance Liquid Chromatography (HPLC) methodology and a chemometric approach to the analysis of the chromatograms.

MATERIALS AND METHODS

Reagents

Chlorogenic acid (5-*O*-caffeoylquinic acid, purity> 95%) and all the other chemicals and reagents of analytical and HPLC grade were purchased from Sigma-Aldrich (Milan, Italy). 3,5-di-*O*-caffeoylquinic acid was isolated and purified from yarrow, and its structure was determined on the basis of NMR and ESI-MS data. The purities exceeded 95% as determined by HPLC analysis (Giorgi *et al*., 2009).

Plant material and aphid infestation

Seeds of the *A. collina* cultivar "SPAK" (Valplantons Bio company; Saillons, Switzerland) were sown in a mixture of standard garden substrate and perlite. Ten days after emergence, they were transplanted in plastic pots containing the same soil and placed in a greenhouse under controlled conditions. *M. persicae* stock cultures were maintained on *Pisum sativum* L. planted in pots filled with perlite and kept in a temperature-controlled glasshouse set at 21°C, under natural light.

The experiments were carried out at a 16 h photophase with 21 °C and a 8 h scotophase with 18 °C, a constant relative humidity of 70%, and daily water supply. Thirty or 50 days after germination, we infested single plants with a colony of about 20-30 individuals. After 10 or 20 days, the aphids were removed with a thin brush, and the leaves of control and infested plants were ground in liquid nitrogen and stored at -80°C until the extraction of the soluble phenolic compounds.

Soluble phenolic extraction

The amount of 0.1 g powdered freezed material of the aerial plant part was processed as follows. The material was mixed with 1 ml of methyl alcohol (MeOH) and macerated under sonication (water bath, room temperature, 30 min). The supernatant was collected after centrifugation. The remaining pellet was re-dissolved in MeOH, and the extraction was repeated. To verify a complete extraction of alcohol soluble phenolics, a spectrophotometric assay by means the Folin-Ciocalteu reagent was applied to each supernatant. The extraction process was repeated as long as phenolic molecules were detected, corresponding to the minimum value of absorbance. All the supernatants of each extract were combined, concentrated at 0.5 ml under vacuum, filtered (0.45 μm MILLEX® HV, Millipore, Milan, Italy) and stored at -20°C. Before injection, the methanol extracts of each sample were diluted with water (HPLC grade), methanol:water (25:75).

Preprocessing: HPLC analysis of phenolic compounds

The extracts were analyzed by using the high performance liquid chromatography (HPLC). Such a technique is commonly applied in the analysis of any class of molecules, including various phenolic compounds. The conditions able to generate meaningful data depend on instrumentation attributes and the method employed in HPLC analysis. The HPLC instrument consisted of a Waters 515 pump connected to a Waters 2487 Dual λ Absorbance UV Detector set at 290 nm (Waters, MI, Italy). Separations were achieved on a reversed phase column C-18 Hypersil ODS (Supelco, particle size 5μm, 250 mm x 4.6 mm) with a C-18 precolumn. The column temperature was maintained at 30°C. The signal-processing programs were obtained from the computer controlled system using upgraded Millenium32 software (Waters, MI, Italy). The results are summarized in a chromatogram, i.e. a sequence of ideally symmetrical peaks which are the detector signals plotted versus the time of separation. The preprocessing seeks standardized conditions for the optimal separation of analytes and good chromatographic peak shape. The optimal separation is the one that, by visual examination, separates best the chromatographic peaks distinguishing between the beginning of a peak and the end of the preceding peak. Such separation was achieved using as mobile phase a solution of 0.05% formic acid in water (solvent A) and methanol (solvent B) at a constant flow rate of 1 ml/min. Initially a concentration of 25% B was maintained for 3 minutes. Solvent B was then increased to 70% at 24 min, 85% at 30 min, 100% at 31 min and finally, isocratic elution with 100% B until 36 min, linear change to 25% B, holding for 10 min to re-equilibrate the column to initial conditions. The injection volume was 20 μl. The hydroxycinnamic acids, previously identified in *A. collina*, such as chlorogenic acid and 3,5-di-*O*-caffeoylquinic acid were injected as external standard solutions.

Preprocessing: validation of HPLC method

According to ICH recommendations (ICH, 2005), the robustness of the method is assessed by validating parameters such as limits of detection and quantification as well as linearity of the analytical procedure. The minimum level at which phenolic components

of *A. collina* could be reliably detected was estimated through the signal-to-noise ratio (S/N) as the limits of detection and quantification calculating the amounts displaying respectively, three (LOD; S/N= 3) and ten (LOQ; S/N=10) times higher, then the baseline noise. The evaluation of the linearity of the calibration response is based on regression and correlation analysis. Specifically, the responses were obtained by plotting the signal, i.e. the areas of the chromatographic peaks against the different concentration of the analytes in the sample (De Backer *et al*., 2009). In this paper, we exclusively report the final result of the evaluation. The validation parameters were qualified by employing six additional calibration levels of chlorogenic acid, and 3,5-di-*O*-caffeoylquinic acid prepared in 25% aqueous methanol ranging from 100 to 0.05 μg/ml were injected in triplicate into the chromatographic system on three different days.

Processing

Treatment 1 was carried out with 30 and 50 days old plants. Plants were individually planted in pots of 0.3 l covered by polyethylene cages. The number of replicates was 20. For treatment 2, the same experimental set-up was used but the plants were infested with *M. persicae* colonies comprising between 20 and 30 individuals that were allowed to feed for 20 days. The number of replicates was 21.

Statistical analysis

The analysis of yarrow's HPLC profiles aims at the identification of the chromatographic peaks separating treatment 1 from treatment 2. The statistical analyses were performed using the Statistical Product and Service Solutions (SPSS) program (Version 18.0; SPSS Inc., Chicago, IL). To illustrate the sequence of the peaks, a chromatogram is presented in Figure 1. To stabilize the variance, the values x of the peaks area were transformed according to $x'=\log(x+1)$. The sequence of peaks in each chromatographic profile provides a time series that is analysed through autocorrelation functions. The Autoregressive Integrated Moving Average Model (ARIMA) is a widely known model for detecting the presence of serial correlation providing a detailed description of the stochastic process. By means of the Ljung-Box, we tested the overall randomness to verify the absence of autocorrelation (Garey *et al*., 2008; Zhao *et al*., 2008).

To identify peaks able to separate treatments 1 and 2, a discriminant analysis was carried out. First, a multiple regression analysis was conducted to assess the collinearity and exclude the peaks that did not satisfied the criteria of both the tolerance (tolerance>0.200) and the Variance Inflaction (VIF<5) which were specified for the SPSS software by Barbaranelli (2009). For this purpose, we employed a stepwise elimination of the peaks. Second, the effect of the remaining peaks in the two treatments was evaluated according to Wilks' lambda statistics (Barbaranelli, 2009). The smaller the value of Wilks' lambda the greater is the contribution to the discriminant function. The assumption of homeoscadisticity was not tested because the number of replicates in each treatment was similar. In this case, the discriminant analysis is robust to the violation of the assumption of homeoscadisticity (Barbaranelli, 2009).

RESULTS

Preprocessing: HPLC analysis of phenolic compounds

The developed HPLC method was able to separate well the phenolic compounds characterizing *A. collina* alcoholic extracts. A separation time of 36 minutes was sufficient to obtain a satisfactory resolution of the substances, and each peak had satisfactory symmetrical shape. The retention times of standard solutions were 9.25 ± 0.16 min and 17.05 ± 0.18 min for chlorogenic acid and 3,5-di-*O*-caffeoylquinic acid, respectively. At these conditions, the HPLC system performs within an acceptable range of precision and linearity.

Preprocessing: validation of HPLC method

Limits of detection (LOD; S/N= 3) and quantification (LOQ; S/N=10) were 0.7 and 5.0 μg/ml for chlorogenic acid and 1.5 and 25 μg/ml for 3,5-di-*O*-caffeoylquinic acid, respectively. Setting the LOD to 0.7 μg/ml for chlorogenic acid, we obtained the corresponding values *x* of the peak equal to 1800 according to the machine specifications. All peaks with area value under 2000 were considered as impurity or noise and disregarded in

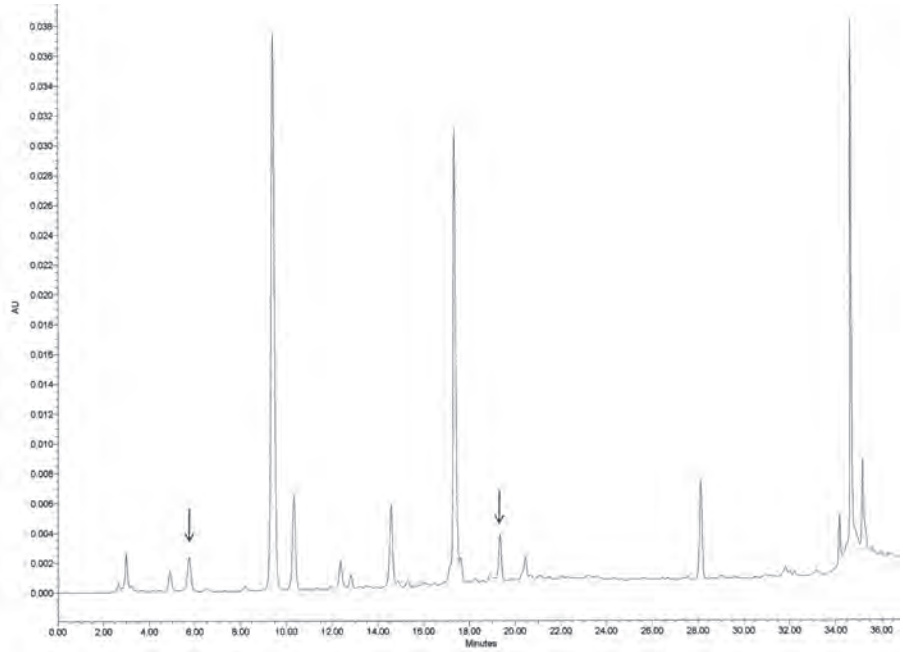

Fig. 1 - The response of *Achillea collina* ex Rchb. to *Myzus persicae* Sulzer infestation: example of chromatogram obtained from aphid infested yarrow leaves (Arrows indicate the discriminatory peaks 6 and 29, the peak obtained after 9 minutes corresponds to chlorogenic acid, the peak obtained after 17 minutes corresponds to 3,5-di-*O*-caffeoylquinic acid).

further analyses. The response appeared to be linear over a wide range of concentrations (100 to 0.05 μg/ml) for each analyte. This is supported by the responses (y) in relation to the concentrations (x), where y = 4980 x + 3.66 with r^2 = 0.999 (chlorogenic acid) and y = 4760 x + 2.23 with r^2 = 0.999 (3,5-di-*O*-caffeoylquinic acid).

Statistical analysis of processing data

The analysis of *A. collina* extracts obtained from experiments A and B provided a matrix with 20 cases for the treatment 1 (not infested) and 21 cases for the treatment 2 (infested). In each case, the chromatographic profiles showed a succession of 58 chromatographic peaks. Figure 1 shows the sequence of well separated peaks in an example of a chromatogram obtained for infested plants and illustrates the patterns that are analyze as follows.

The Autoregressive Integrated Moving Average Model (ARIMA) applied to the different samples produced only occasionally significant autocorrelations with no consistent patterns. Therefore, we disregarded the autocorrelation in further analysis.

Tab. 1 lists the 20 peaks, out of 58, that meet the tolerance criteria of collinearity in the stepwise regression analysis.

Tab. 1 - The response of Achillea collina *ex Rchb.* to Myzus persicae *Sulzer infestation: tolerance values of the predictor variables, reported as peak number with retention time (Rt).*

Peak number	Rt (minutes)	Tolerance
3	3.25	0.450
6	5.86	0.330
13	9.25	0.266
15	10.29	0.288
16	10.42	0.449
18	11.60	0.386
19	12.05	0.419
20	12.22	0.292
21	12.62	0.326
23	13.36	0.496
29	19.34	0.437
33	20.13	0.282
44	25.89	0.316
48	27.93	0.417
50	28.42	0.489
51	29.00	0.342
52	29.42	0.362
53	30.19	0.455
54	31.04	0.370
55	31.21	0.423

The canonical discriminant coefficients are 0.831 for peak 6 and 0.688 for peak 29. The Wilks' Lambda value equal to 0.01 indicates a good discrimination capability (Barbaranelli, 2009). Accordingly, peak 6 and peak 29 with retention times of 5.86 and 19.34 minutes have a significant discriminatory effect. These two peaks have been marked in Figure 1.

Tab. 2 reports the predictive capability of the discriminant model. Accordingly, 73.2% was the overall hit ratio, representing the percentage of original treatments (not infested and infested). 76.2% was the correctly classified control cases while the 70.0% was the correctly classified infested cases.

Tab. 2 - The response of Achillea collina *ex Rchb. to* Myzus persicae *Sulzer infestation: classification of the peaks obtained in the chromatograms*[a].

Treatment			Predicted Group Membership		Total
			1.00	2.00	
Original	Count	1.00	16	5	21
		2.00	6	14	20
	%	1.00	76.2	23.8	100.0
		2.00	30.0	70.0	100.0

a. 73.2% of original grouped cases correctly classified.

DISCUSSION

Secondary metabolites play a major role in the adaptation of plants to their environment (Ehrlich *et al*., 1964; Wink, 2003; Benderoth *et al*., 2006). Recently, Futuyma & Agrawal (2009) described the role of secondary metabolites in evolution theories, biodiversity and plant defense. This paper is restricted to the response of the plant in terms of phenolics and disregards other biochemicals and biophysical elements (Schowalter, 2006). Moreover, the treatment combines infestations obtained with colonies of different size feeding on the plant over 10 or 20 days time interval and compares the results with a control. More detailed information on colony sizes and the definition of precise feeding times may further support the results of these analyses. Anyway, the results of this study may contribute to a better understanding of the effect of phloem feeding on plant biochemistry. Although secondary metabolites are not directly implicated in vital cell functions such as respiration and division, they are a subject of considerable interest, particularly because of their utility in human medicine and nutrition. They have been widely studied by plant biochemists who attribute to them physiological, agronomic and ecological importance (Bennett & Wallsgrove, 1994; Pichersky *et al*., 2000; Bourgaud *et al*., 2001; Wink, 2003). The wide diversity of chemical structures is related to a multiplicity of functions. Among the different secondary metabolites, the phenolic compounds stand out because of their capability to defend plants from herbivores (Schowalter, 2006). The literature reports that the phenolics have deterrence properties and toxicity against different species of insects (Miles *et al*., 1999; Appel,

1993; Golawska *et al*., 2009). Our analysis of the yarrow system started with phloem feeding aphids rather than leaf feeding chrysomelids which are also present in yarrow fields (Morlacchi, 2010). *M. persicae* can easily be reared on yarrow and proved to be an ideal model of a phloem feeder for studying plant-aphid interactions (Morlacchi, 2010). Presumably, the here described processing procedure could be applied to study the response of the plant to chrysomelid feeding.

The literature provides guidance on the selection of instruments, the chemometric methods and the interpretation of the results (Jiao *et al*., 2010; Rodriguez-Nogales, 2010). In this paper, we carried out preprocessing to obtain the optimal separation of the analytes, even when present in small amounts. The methanol extract of *A. collina* is a complex pool of molecules and the corresponding chromatographic profiles showed a sequence of overlapping peaks. Thus, the preprocessing required great effort to find the best chromatographic conditions that separate phenolic compounds. Finally, the preprocessing procedure resulted in a satisfactory methodology fulfilling the criteria of optimal separation of analytes, good chromatographic peak shape and robustness of the results. This allowed us to directly proceed to the processing phase and carry out chemometric analyses with a standardized methodology. This step is considered important for an efficient identification of peaks separating infested from non-infested plants. It does not compromise the possibility for further refining preprocessing procedures (Hendriks *et al*, 2005) and re-analyses of the here obtained results.

Chemometric analyses are widely used to extract information from chemical system (Bereton, 2003; Jiao *et al*., 2010; Rodriguez-Nogales *et al*., 2010). A stepwise multivariate analysis is a powerful technique for identifying the difference between treatments with respect to several variables and for classifying the discriminatory compounds. Hydroxycinnamic acids previously found in *A. collina* have no significant discriminatory effects. This result is in contrast to the one obtained by other authors (Miles *et al*., 1999; Cipollini *et al*., 2008), which have found that chlorogenic acid and other caffeic acid derivatives increased during feeding of other insects species. The contrast is probably due to the different mechanisms that plants activate in relation to the herbivore feeding behavior (Hao *et al*., 2008). Hopkins *et al*., (2009) explained the role of glucosinolates as defensive compounds and reported a huge variation in the biological activity of individual compounds suggesting that the total secondary metabolites concentration is not able to explain exhaustively their biological effects. Moreover, the authors reported the qualitative and quantitative variation respect to the plant tissues and ontogenetic stages and the extreme variation in the role that such secondary metabolites play for different insects and herbivores.

The most important finding is that aphid feeding changes the chemical composition of the host plants. Specifically, we obtained two peaks (6 and 29 in the chromatogram) able to separate infested from non-infested plants. The coefficient value of the peak 6 was higher respect to those of the 29, suggesting a major discriminatory capacity of this compound. Since the extraction procedure and the HPLC method were specific to phenolic compounds, we suppose that the molecules corresponding to 6 and 29 peak belong to this specific chemical class. Phenolic compounds are distributed in different

tissues and subcellular leaf structures. It is of particular interest to evaluate a possible use in pest management systems aiming at phloem feeding insects (Beninger et al., 2004; Ranger et al., 2007). The work shows that the plant responds with the production of two unknown phenolics to aphid infestation. Nevertheless, the exact nature of the chemicals is currently unknown, and work is under way to identify the substances responsible for producing these peaks. To further improve the insight into the response of the plant to phloem feeding, efforts should be made to localize the production within the plant. Morevover, the work deals exclusively with the response of the plant to aphid infestation. The effect of phenolics and other substances on aphid development will be treated in a subsequent paper.

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