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DAMAGES ON *FRAGARIA ANANASSA* VAR. 'SNOW WHITE' CAUSED BY *ERWINIA AMYLOVORA* AND POTENTIAL OF *CHAENOMELES* EXTRACT FOR DISEASE CONTROL

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Abstract

Plant pathogens present serious challenges before producers. Erwinia amylovora is a polyphagous pathogen that also affects strawberries. However, data on bacterial pathogens on this crop is scarce worldwide and lacks in Bulgaria. The present study aimed to investigate the damages that E. amylovora induces in greenhouse-grown strawberries (Fragaria ananassa) from the variety "Snow white" in Bulgaria, with respect to different irrigation and fertilization levels, and the potential of a plant-derived extract from fruits of Chaenomeles sp. to be used for disease control. The extract has previously shown to have good antibacterial activity and it exhibited no phytotoxicity on the strawberry plants. The results showed raised leaf losses for the inoculated with pathogen vs the non-inoculated plants and vs the inoculated and treated with extract plants. The flower losses due to infection are significant and the inoculated plants lose about 2.3 times more flowers compared to the non-inoculated plants and about 2 times more flowers compared to the inoculated and treated plants, thus revealing a clear tendency towards flower damaging caused by the pathogen and a protective effect of the used extract.

Key words*: Erwinia amylovora, Fragaria ananassa, disease control, Chaenomeles sp., plant extract.*

INTRODUCTION

Plant pathogens present serious challenges before producers. *Erwinia amylovora* is a polyphagous pathogen that poses a major threat worldwide, infecting, spreading and surviving on an extremely large number of plant species. These factors make this devastating disease difficult to control. Copper preparations and antibiotics are still the most effective solutions to combat the pathogen, although their use contributes to environmental pollution and the development of resistant populations. In Europe, the use of antibiotics for agricultural purposes is prohibited, due to considerations of increasing antibiotic resistance among medical strains of pathogens and the protection of human health. Therefore, the need of new plant protection products is still urgent and pressing.

Plants synthesize secondary metabolites, some of which are highly active against certain pathogens, and offer promising prospects for alternative plant disease control and bioproduction. The scientific interest in these natural sources of potential control agents is not waning. Plant extracts have been investigated for antimicrobial properties against pathogens, but still from a very limited number of plant species (Stangarlin et al., 1999; Schwan-Estrada et al., 2005; Mohamed, 2010; Stoyanova & Valkova, 2014; 2015a; 2015b). The limited data is mainly due to several elements: a large number of pathogens, local strain differences, a huge number of plant species that could be tested, different extraction methods with corresponding differences in the composition of the finished extracts and potential differences in the methods of application and/or the concentrations used. Most investigations are *in vitro* studies and chemical analyses, but experiments with living plants are extremely insufficient. The reasons are different, and in some cases the main problem is the host crop of a certain pathogen. The nature of fruit tree species, which suffer the most from *E. amylovora*, is a major obstacle to experimental

work. Experiments with orchards require large areas, specific isolation requirements, years for trial preparation, industrial quantities of the tested candidate drug and potential eradication of the orchard at the end of the study. A potential solution to the problem is the discovery of plant-based products with activity against the causal agent, development and optimization of their application on more convenient for cultivation, treatment and monitoring host crops, such as strawberries. This approach can be compared to the practice in pharmacy and medicine, where research on potential drugs is tested on mice before moving on to testing on volunteers. On the other hand, the discovery of a biological agent for controlling fire blight of strawberries would be beneficial itself given the way of consumption of the fruits - after simply washing with water without the possibility of "scrubbing" the surface or peeling. Data on bacterial pathogens on strawberries is scarce worldwide.

Some plant extracts have shown activity against *E. amylovora in vitro* (Stoyanova & Valkova, 2014; Stoyanova et al., 2024). A promising option for alternative biocontrol of the pathogen presents the extract from *Chaenomeles* sp. fruits. The plant species is unpretentious, native for Asia and introduced in many European countries. Extracts from several genotypes have been tested, all of which possessed antibacterial activity against *E. amylovora* (Stoyanova et al., 2024). The experiment in this study was based on the hypothesis that preventive spraying of host plants with the plant extract may reduce the pathogen population on the leaf surface and thus, to prevent its entry into plant tissues and to reduce the development of disease symptoms on plants.

The study aimed to investigate the damages that *E. amylovora* induces in greenhouse-grown strawberry plants (*Fragaria ananassa*) from the variety “Snow white”, with respect to different irrigation and fertilization levels, and the potential of a plant-derived extract from fruits of *Chaenomeles* sp. to be used for disease control.

MATERIALS AND METHODS

Plant material: Fruits from *Chaenomeles* sp. plants genotype 6’ and genotype 29 originating from the field of the Research Institute of Mountain Stockbreeding and Agriculture, Agricultural Academy, Troyan, were used in this study. The fruits were collected in the autumn of the year prior to the experiment and stored freezed at -20°C.

Extractions: Extracts were prepared from smashed fruits in methanol (HPLC grade) using Soxhlet at 80°C for 5 hours. The solvent was separated in vacuum vaporizer (model Hei VAP Precision, Heidolph Inst.) at 55°C, 30 000 Pa. The extracts were concentrated at decreasing pressure up to 7 200 Pa and increasing temperature up to 70°C. Storage of the extracts was in brown bottles, at 18°C, in the dark. Working solutions of the extracts was 2% (w/v, in water), freshly prepared 1 h before the treatments. The extracts were applied by spraying. Choice of dilution was based on a previous study for *in vitro* antibacterial activity and minimal inhibitory concentration of extracts from the two genotypes (Stoyanova et al., 2024).

Bacterial strains: *Erwinia amylovora* NBIMCC 2331 (=ATCC 15580, type strain, isolated from pear) and NBIMCC 8473 (Bulgarian strain from *Fragaria ananassa*) were used for inoculations of the plants. Bacteria were applied as water suspensions with transparency 65% (NBIMCC 2331) and 75% NBIMCC 8473 (measured with turbidimeter Biolog Inc.) by spraying.

Test plants: A total of 153 strawberry plants *Fragaria ananassa* variety 'Snow White' grown in a non-heated polyethylene tunnel greenhouse, located at Experimental base “Chelopechene” of the Institute of Soil Science, Agrotechnologies and Plant Protection “Nikola Pushkarov”, Agricultural Academy, Sofia, Bulgaria (latitude 42°44′22.8″N, longitude 23°28′3.7″E and altitude 550 m above sea level), were subjects of this study. The plants were drip irrigated and mulched with silver-black UV polyethylene film with a thickness of 30 μm. Covering of the greenhouse was with a five-layer UV+EVA+IR+AD+dif -150 μm polyethylene film.

Phytotoxicity tests: During the growing season, prior to the main experiment, a preliminary investigation to establish possible phytotoxicity of the used extracts on white strawberry plants in 2 replicates was carried out. In the first replicate, strawberries were grown in pots with a diameter of 30 cm and 10 L of soil at outdoor temperatures, outdoor humidity and artificial

irrigation, and in the second - in pots with a diameter of 30 cm and soil 10 L of soil under outdoor conditions. The test plants were divided into four lots with five plants per lot for each of the two extracts: 1) non-treated (unsprayed) plants; 2) non-treated plants sprayed with water; 3) treated plants, sprayed with 2% extract; 4) treated plants, sprayed with 5% extract. Treatments were applied 3 times every 7 days.

Treatments: The experiment included two consecutive rounds, each including: a) treatment, letting dry for 1 h, inoculation; b) treatment every 7 days for the next 3 weeks. The first round of the experiment was performed with genotype 6' extract and the type strain of the pathogen. The second round was performed with genotype 29 extract and the strawberry strain of the pathogen. The observations were carried out every week before the treatments. The numbers of withered flowers and leaves of each plant were recorded and the same were removed from the plants and the greenhouse to avoid recounting.

Variants of irrigation and fertilization (VIF): We used 5 VIF: 100% irrigation (I0F0), 50% irrigation and 100% fertilization (I2F1), 75% irrigation and 100% fertilization (I1F1), 75% irrigation and 75% fertilization (I1F2), 50% irrigation and 75% fertilization (I2F2).

Setting of the experiment: For each VIF, plants were divided into three groups: treated non-inoculated (negative control, healthy), treated and inoculated with pathogen (experimental) and inoculated only (positive control, infected). 10±3 plants were used for each option. The study was conducted in the period May-June of 2024.

Statistics: The collected data were statistically analyzed by Duncan's Multiple Range tests at significance levels $p<0.05$ and $p<0.01$ using STATISTICA 8.0.

RESULTS AND DISCUSSIONS

In the preliminary phytotoxicity tests, no negative changes were observed. The leaves of the treated plants did not exhibit any changes in appearance and there were only single withered flowers in all pots with similar count. The overall development of the plants was good and similar in all pots, so it could be concluded that the extracts from *Chaenomeles* were not phytotoxic to strawberry plants.

The symptoms of infection with *E. amylovora* on the leaves of white strawberries in the greenhouse were mainly in the form of marginal necrosis of the young leaves, although diffuse necrosis and chlorosis could also be observed. Necrosis on flower stalks and sepals was very common (Figure 1).

Figure 1. Symptoms of fire blight on leaves and flowers of *F. ananassa* var. 'Snow White'

When considering the total number of dead leaves and flowers (for all VIFs), the highest number of dead leaves was observed in the inoculated non-treated (infected) plants in both rounds of the experiment (Figure 2). In the treated plants (both inoculated - experimental, and non-inoculated - healthy), no dependence was observed in the loss of leaves when comparing the two consecutive experiments - in the second round the experimental plants lost 39% less leaves and the number was also lower compared to the healthy plants. The infected plants also lost less leaves (Figure 1). For the total period of 8 weeks, the results show an increased leaf loss by ~47% of the diseased plants compared to the healthy ones and by ~21% compared to the experimental ones. Much greater differences were observed in the loss of flowers: the diseased plants lose ~2.3 times more flowers compared to the healthy plants and by ~2 times more compared to the experimental plants (Figure 2).

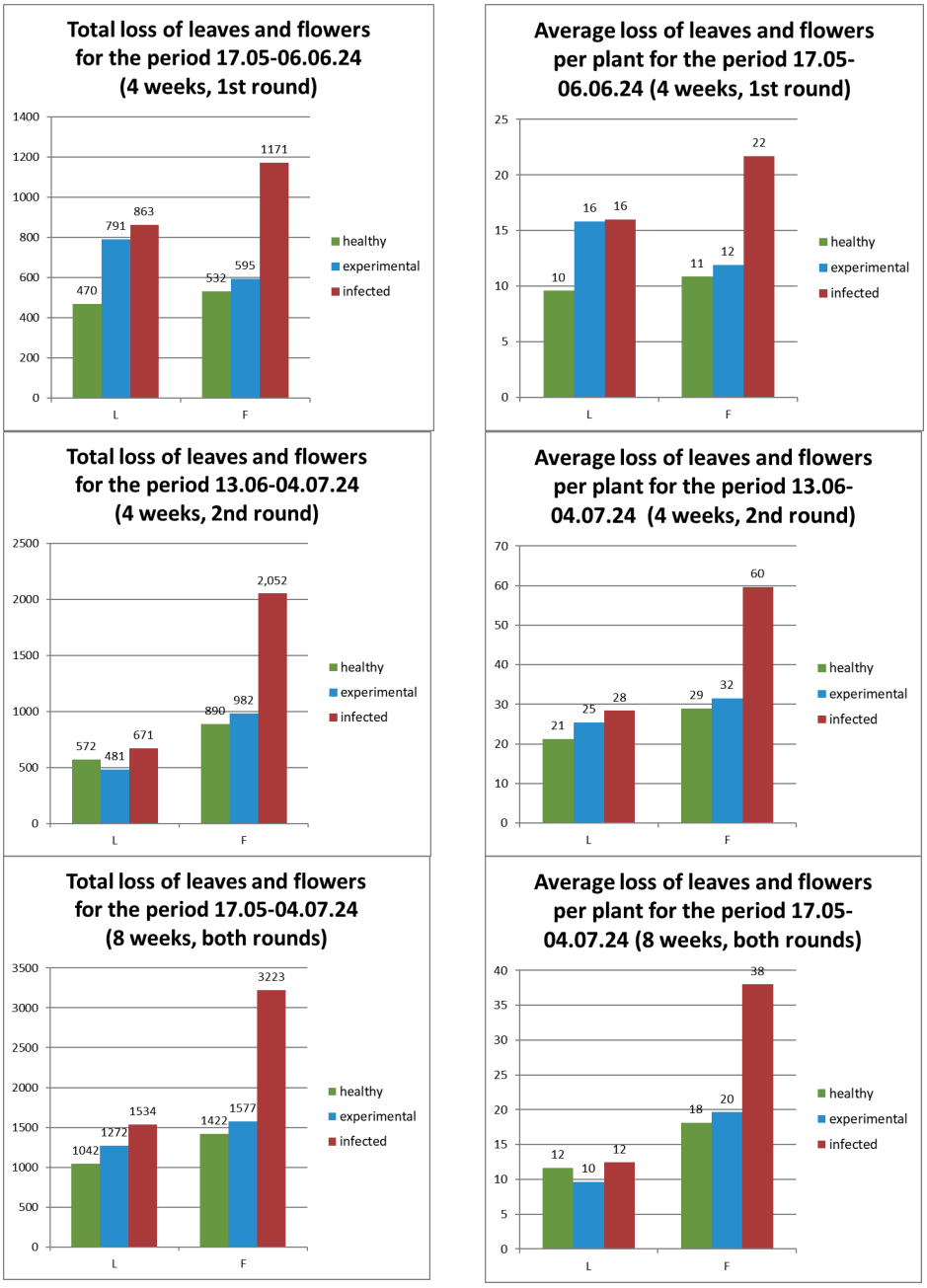

Figure 2. Loss of leaves (L) and flowers (F) of treated, experimental and diseased plants

Flower loss followed the same trend in the both rounds of the experiment - the ratios among the three groups are very similar (Table 1).

Table 1. Approximate ratios of leaf and flower loss between the two rounds of the experiment (values are rounded)

	H: E: I	
	leaves	flowers
1 round	2.5: 4: 4	1.1: 1.2: 2.2
2 round	2.6: 3: 3	1: 1.1: 2

Distribution among VIFs of leaf loss did not follow a clear trend (Figure 3). Greatest leaf loss was observed for infected plants from variants I1F1 and I1F2, which are the richest combinations between irrigation and fertilization. These are also the only two VIFs, at which the plants lost less leaves if treated compared to the infected plants. At the variants with the most restricting conditions I2F1 and I2F2 leaf loss was intricate. At I0F0 leaf loss of experimental plants is similar to the infected ones (Figure 3).

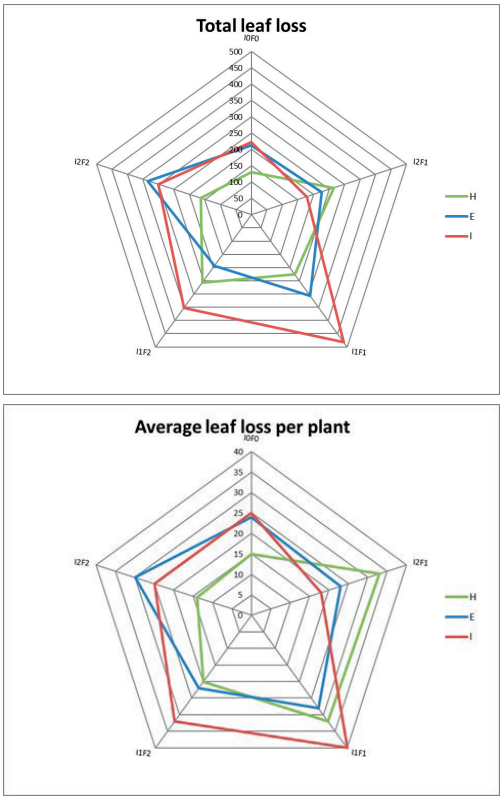

Figure 3. Leaf loss by variants (VIFs)

The loss of flowers was much greater and followed a certain trend - the counts were always the highest for the infected only plants (Figure 4). Greatest leaf loss was observed for infected plants from variants I1F1 and I2F2, which are the richest and the most restricting combinations, respectively, between irrigation and fertilization. At all variants leaf loss was greatly reduced for the experimental plants (Figure 4).

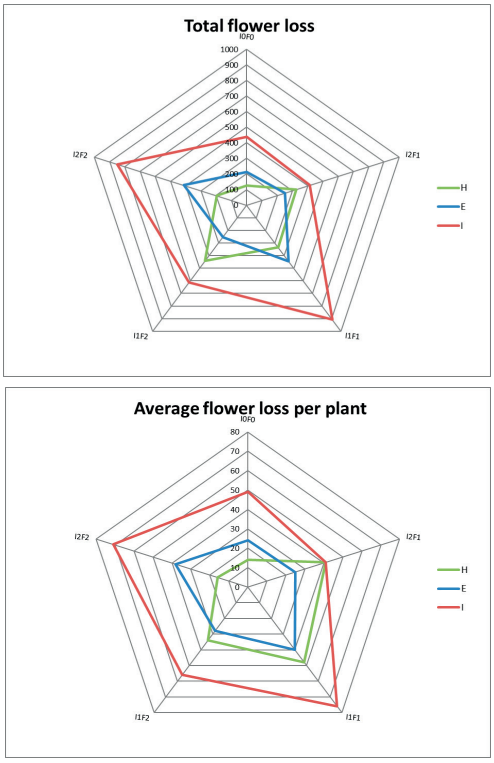

Figure 4. Flower loss by variants (VIFs)

At this point, the richest combination of irrigation and fertilization I1F1 seems to favor the development of fire blight in means of leaf and flower loss and the treatments with chaenomeles extract was able to lower these losses.

Distribution among VIFs of flower loss (with the exception of I2F1) showed a repetitive tendency between the two rounds of the experiment: I0F0 and I2F2 plants (grown at the most water favorable and the most unfavorable conditions) lost the most flowers if infected and the least - if healthy, with the experimental ones positioning in the middle. In the other variants, the experimental plants lost less flowers compared to the negative control (Figure 5).

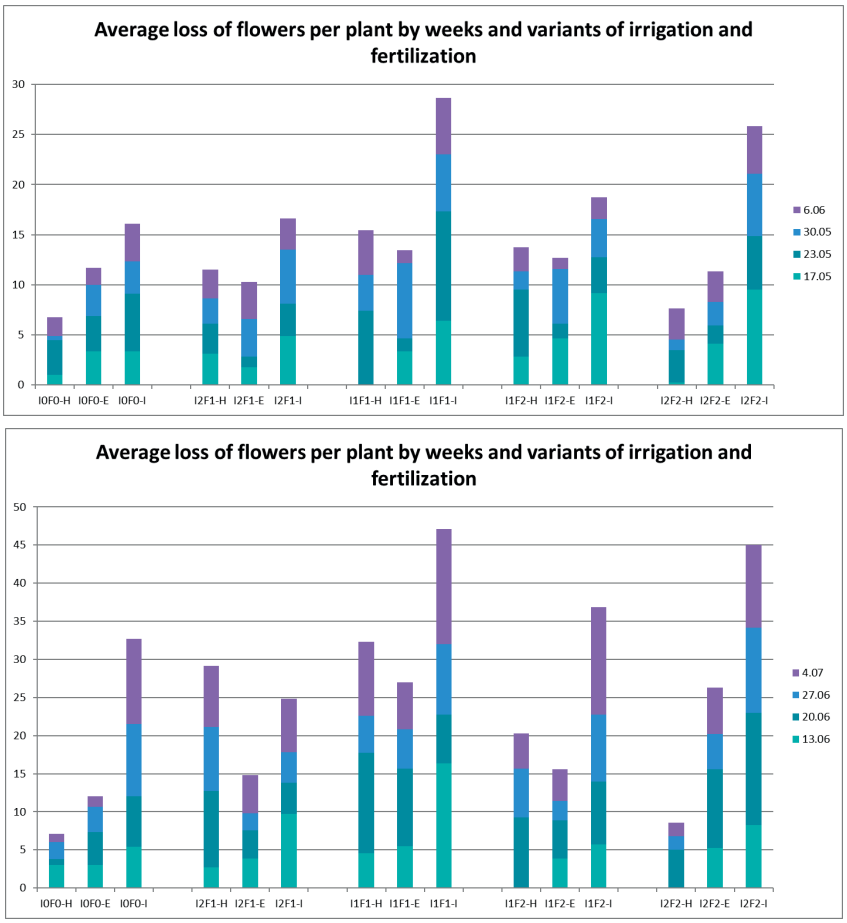

Figure 5. Average flower loss per plant by variants (VIFs)

Statistical analysis of all collected data for leaf loss showed significant differences ($p<0.05$) among all 15 variant groups (experimental groups with regards to the VIF), although only I0F0-H, I2F2-H (the healthy plants, grown at the most water favorable and the most restricting conditions) and I1F1-I are clearly separated from all others (Table 2).

Table 2. Average loss of leaves of treated, experimental and infected plants with regard to VIFs

Leaves	Healthy	Experiment	Infected
I0F0	7.28a	11.78ab	12.33abc
I2F1	16.56bc	11.30ab	8.90ab
I1F1	16.07bc	13.91abc	20.04c
I1F2	9.88ab	10.78ab	16.05bc
I2F2	6.83a	15.12bc	12.50abc

Values with same lowercase letter for the same parameter were not statistically different.

There were no statistically significant differences in leaf loss among the three groups (healthy-treated, experimental-treated inoculated and infected-inoculated) in each round of the experiment as well as among the VIFs.

Statistical analysis of all collected data for flower loss showed significant differences ($p<0.01$) among all 15 variant groups (experimental groups with regards to the VIFs), although only I0F0-H (the healthy plants, grown at the most water favorable conditions) and I1F1-I (the infected plants from the richest combination of VIF) are more clearly separated from all others (Table 3).

Very high statistical significance $p<0.001$ was observed among the groups in flower loss: the infected plants differentiate from the healthy and experimental ones (Table 4).

Table 3. Average loss of flowers of treated, experimental and infected plants with regard to VIFs

Flowers	Healthy	Experiment	Infected
I0F0	6.94a	11.83abc	24.39cde
I2F1	20.31bcd	12.55abc	20.7bcd
I1F1	23.86cde	20.23bcd	37.875f
I1F2	17.04abcd	14.11abc	27.77def
I2F2	8.13ab	18.82abcd	35.42ef

Values with same lowercase letter for the same parameter were not statistically different.

Table 4. Average loss of flowers of the groups

Experiment	Flowers
healthy	15.26a
experiment	15.51a
infected	29.23b

Values with same lowercase letter for the same parameter were not statistically different.

There was also a significant (p<0.01) difference between the two rounds of the experiment due to the much greater flower loss during the second round. However, the ratio of the values between the three groups is similar (Table 1). Three factors can have an effect on the experimental results – the pathogen strain, the genotype used for extraction and the temperature range during the experimentation period. Since the trend of losses between the two rounds is the same (losses increased in all the three groups with a similar ratio) it is very unlikely that the strain or the genotype exerted great effects, since both factors influenced only two of all three groups. At the same time, temperatures during the second period are much higher (Table 5) and they influence all the plants. The maximum temperature of the second period is 4°C higher compared to the first period and a gradual rising trend of the maximum and the average temperatures during the weeks of the first period can be observed. Higher temperatures can have an additive effect to the physiological leaf and flower loss.

Table 5. Basic temperature data during the experimental period (average data, °C)

Week	09.05-17.05	18.05-23.05	24.05-30.05	31.05-06.06	Average ±SD
Min.	4.82	9.46	7.80	11.03	8.28±2.66
Max.	38.06	43.13	44.94	47.71	43.46±4.06
Average	16.28	20.36	21.93	27.29	21.46±4.55

Week	07.06-13.06	14.06-20.06	21.06-27.06	28.06-04.07	Average ±SD
Min.	13.93	9.24	16.94	11.10	12.80±3.37
Max.	49.51	50.89	51.70	48.91	50.25±1.27
Average	29.21	28.67	29.90	28.23	29.01±0.72

Round	09.05-06.06	06.06-05.07	variance
Min.	4.82	9.24	4.42
Max.	47.71	51.70	3.99
Average	21.14	29.02	7.87

The flower losses in the two rounds of the experiment reveal the real effect which the pathogen *E. amylovora* exerts on strawberry plants. Though the loss of leaves is not so obvious, the infection progresses in a "sneaking" way causing significant flower loss, which can be missed below the surfaces of the leaves. At the same time, treatments with *Chaenomeles* extract lower these losses to levels that are comparable to the healthy plants

CONCLUSIONS

E. amylovora can cause a "sneaking" infection on strawberry plants *F. ananassa* var. 'Snow White', grown in greenhouse, causing significant loss of flowers. Water solution of concentrated *Chaenomeles* extract can exert a protective effect against the infection with the pathogen, lowering the flower loss to levels comparable with these of the healthy plants.

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