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Microbial Organic Compounds (MVCs) as an Inducer of Strawberry Plant Resistance to *Strawberry Vein Banding Virus* (SVBV) Infection

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Abstract

Strawberry vein banding virus (SVBV) is a plant virus that greatly affects the quantity and quality of strawberry plants. An alternative method in controlling SVBV is to increase plant resistance induced by *Microbial Organic Compounds* (MVCs) produced by the bacterium Bacillus coagulans. The results showed that B. coagulans treatment that produced 28 MVCs was able to reduce disease severity based on symptom development and reduce virus accumulation in strawberry plants based on NAE. Identification of antiviral compounds by GC-MS showed that strawberry leaves inoculated with SVBV in B. coagulans treatment contained antiviral compounds cytosine riboside (1.53%), palmitic acid (15.9%), linolenic acid (41.81%), and 1-naphthalenesulfonic acid (6.46%). The results of this study indicate that B. coagulans is able to induce systemic resistance to SVBV infection in strawberry plants.

Keywords: Induction of MVCs, antiviral compounds, strawberry plant

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1. Introduction

Strawberry Vein Banding Virus (SVBV) is a virus that belongs to the genus Caulimovirus (retroid virus), has a dsDNA genome, and can be transmitted by aphids in a semi-persistent manner (Vaskova et al., 2004). SVBV causes vein banding and chlorosis symptoms in strawberry plants and has been found in North America, Australia, Brazil, Japan, Europe, China (Feng et al., 2016) and Bali (Sudiarta et al., 2021).

One of the plant virus control technologies is Induced Systemic Resistance (ISR) technology induced by Bacillus coagulans bacteria that produce *Microbial Organic Compounds* (MVCs). Each bacterial strain has a different ability to increase plant resistance through the ISR mechanism. Ramamoorthy et al. (2001) reported that ISR is plant resistance that occurs due to changes in plant biochemistry or physiology which then stimulates the formation of PR proteins (pathogenesis related proteins), phytoalexin synthesis, and other secondary metabolites. Xing et al. (2020) reported that Bacillus simplex Snap 545 treatment in soybean plants was able to reduce the development of symptoms caused by Heterodera glycines infection, B. simplex Snap 545 produces cyclic compounds (Val-Pro), tryptophan, and uracil which induce soybean plant resistance through salicylic acid and jasmonic acid pathways. Abdelkhalek et al. (2022) reported that the treatment of Bacillus amyloliquefaciens strain TBorg1 which produced MVCs 1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester, phenol, 2,4-bis(1,1-dimethylethyl), L-proline, N- valeryl-, and heptadecyl ester in tomato plants was able to increase plant resistance to Tobacco mosaic virus (TMV) infection through the formation of polyphenolic compounds and the expression of pathogenesis-related genes (PR-1 and PR-5). The aim of this study was to use a suspension of B. coagulans that produces *Microbial Organic Compounds* (MVCs) to induce strawberry plant resistance to *Strawberry vein banding virus* (SVBV) infection.

2. Research Methods

This research was carried out with several stages including making bacterial cultures, virus inoculation, GCMS detection, ELISA detection and measuring leaf chlorophyll levels. Mechanical virus inoculation activities were carried out at the Plastic House in Bedugul, Candikuning Village, Baturiti District, Tabanan Regency. Meanwhile, the activities of making B. coagulans culture, detection of MVCs using GCMS, and detection of SVBV by ELISA and determination of leaf chlorophyll levels were carried out at the Plant Disease Laboratory, Agroecotechnology Study Program, Faculty of Agriculture, Udayana University.

2.1.Establishment of B. coagulans culture

B. coagulans bacteria were grown in Peptone potato dextrose agar media (10 g peptone, 100 ml potato extract, 20 g dextrose, 20 g agar) for 48 hours and incubated at 35 oC. A total of 10 ml of B. coagulans culture suspension at a density of 2 x 108 CFU was poured onto 1 month old strawberry plants. Meanwhile, for the control treatment, strawberry plants were watered with 10 ml of sterile water.

2.2 Mechanical virus inoculation

SVBV inoculation was carried out 15 days after the bacterial flushing treatment. Sap made from plants positively infected with SVBV was crushed in a sterile mortar and mixed with 0.01 M phosphate buffer solution (pH.7) with a ratio of 1 g of SVBV infected leaves per 5 ml of phosphate buffer solution (1:5 w/v). Sap is inoculated into the two youngest leaves that have fully opened. Before inoculation, carborundum is sprinkled on the upper surface of the leaves, then the sap is applied with sterile cotton wool to the surface of the leaves starting from the lower leaves to the upper leaves in the same direction without repeating on the same area.

2.3 Detection of MVCs by Using GCMS

Identification of MVCs in the bacterial filtrate of B. coagulans using a GCMS Agilent 6980N Network GC System with an Agilent 5973 inert MSD detector (70 eV direct inlet) according to the method of Kannan et al. (2016). 2 µl of bacterial filtrate sample solution was injected into the GC MS which has a J&W Scientific, HP-5MS capillary column with a length of 30 mm, a diameter of 0.25 mm and a thickness of 0.25 µm. Helium carrier gas at a flow rate of 1 ml/minute (constant) with a split ratio of 1:10. The programmed oven temperature was 500C and kept isothermal for 5 minutes, the rate increased to 10 0C/minute and the temperature was increased to 2800C for 15 minutes. the injector port temperature is 2900C and the mass spectrometer is 2300C. Identification of MVCs uses the Willey database version 7.0 by comparing mass spectrum patterns and fragmentation patterns of reference compounds stored in the Wiley library.

2.4 Detection SVBV with ELISA and Determination of Leaf Chlorophyll Content

SVBV detection was carried out 2 weeks after inoculation using the DAS-ELISA (Enzyme-Linked immunosorbent Assay) serology method using SVBV antiserum. The DAS-ELISA results were analyzed quantitatively with an Elisa reader (Bio-RAD model 550 microplate reader, Tokyo, Japan) at a wavelength of 405 nm. 1g of strawberry plant leaves infected with SVBV and not infected with CMV were homogenized with a mortar and 1 ml PBS (10 nM N2HPO4, 0.1 M NaCl, pH 7.0) 1:10 (g:ml). The homogenate was filtered with filter paper and the filtrate was collected. The wells of the ELISA plate were coated with anti – SVBV immunoglobulin g (IgG) at 1.5 µg/ml in buffer (35 mM Na2HCO3, 15 mM Na2CO3, 0.2% serum albumin (BSA), and 2% polyvinylpyrrolidone, pH 9.6 , 100 1µ/well) and incubated at 4°C for 12 hours. The plates were washed three times as before. conjugated IgG 1µ/well). The plates were incubated at 37°C for 1 hour. Plates were washed as previously described and subtract P-nitrophenyl phosphate at 1mg/ml in 10% diethylamine pH 9.8 supplemented (100 1µ/well) and absorbance value at 405 nm. A sample is declared positive if the ELISA absorbance value (NAE) of the test sample is 2 times greater than the NAE of the ELISA negative control (healthy plant). Leaf chlorophyll content (SPAD units) was determined using a Chlorophyll-meter SPAD-502 (Konica Minolta, Japan).

3. Results and Discussion

The results showed that watering treatment with 10 ml of B. coagulans culture suspension per strawberry plant was able to reduce the number of SVBV particles significantly (Figure 1). This is proven by the NAE in strawberry plants treated with B. coagulans being lower compared to the NAE in the control treatment. The NAE in strawberry plants treated with B. coagulans was 0.023, while the NAE in the control treatment was 3.765.

Strawberry plants that were treated with watering with 10 ml of B. coagulans culture suspension per plant were able to reduce the development of SVBV symptoms, this was proven by the value of leaf chlorophyll levels in strawberry plants treated with B. coagulans being higher when compared to the value of leaf chlorophyll levels in control plants. The value of leaf chlorophyll content in strawberry plants treated with B. coagulans was 39.75 SPAD, while the leaf chlorophyll content of strawberry plants in the control treatment was 27.56 SPAD.

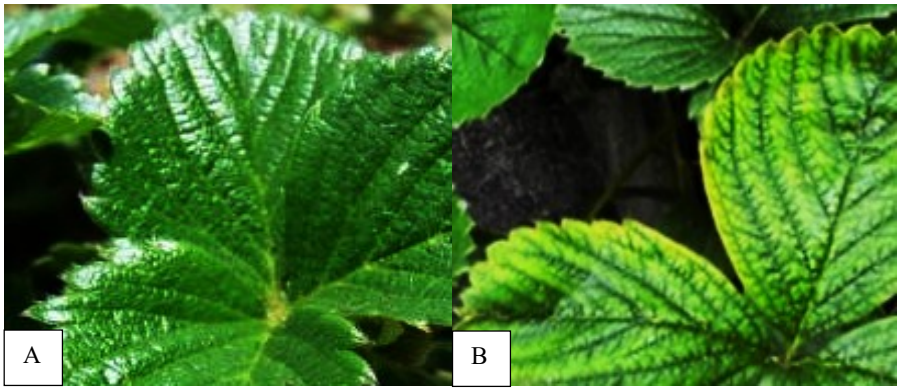

Figure 1. Strawberry plant performance. **(A)**. strawberry plants in the B. coagulans treatment (SVBV negative): **(B).** strawberry plants in the control treatment (SVBV positive).

The watering treatment of 10 ml of B. coagulans culture suspension per plant was able to induce resistance in strawberry plants by reducing the severity of the disease based on the development of symptoms, and reducing virus accumulation in strawberry plants based on the ELISA absorbance value at 405 nm. B. coagulans treatment was able to reduce symptoms caused by SVBV infection and reduce virus accumulation in plants based on NAE, namely from 3.765 to 0.023. The results of this research are in line with the results of research by Al Ani and Adhab (2012) who reported that seed treatment and watering with P. fluorescens suspension at a density of 4 x 109 was able to reduce CMV accumulation in melon plants based on the ELISA absorbance value at 405 nm. Latake and Borkar (2017) reported that cucumber seeds soaked in Streptomyces olivaceus filtrate for 8 hours were able to reduce CMV symptoms.

The mechanism of B. coagulans in reducing symptoms and virus accumulation in plants is through the induction of systemic resistance by means of MVCs produced by B. coagulans inducing the formation of antiviral compounds in strawberry plants. Based on the results of GCMS analysis, it shows that the B. coagulans filtrate contains 28 MVCs (Figure 2). Twenty-eight MVCs were hexanal; cyclopentanol, cyclopentanone, 3-methyl; propanoic acid, 2-hydroxy; benzene, 1,4-dimethyl; ethanol, 2-2-aminoethoxy; acetic acid, anhydride; ethanol, 2-2-aminoethoxy; acetaldehyde, dihexyl acetate; 1-hexyl iodide; propanal, dimethylhydrazone; urea, 1,1-dimethyl; pentane, 2,3,3,4-tetramethyl; 3-1,3-dimethylbutoxy-2-butanol; cis-2-buteneoxide; ethylele glycol monohexyl ether; neoheptanol; isoheptane; sulfurous acid, isohexyl 2-propyl; sulfurous acid, isohexyl 2-propyl; ethyl cyclohexyl ketone; tryptene; oxalic acid, cyclohexyl tetradecyl; oxalic acid, cyclohexyl tetradecyl; allylacetone; 1-cathinone; 1H-indole-3-ethanamine; decamethyltetrasiloxane; and decamethyltetrasiloxane (Table 1).

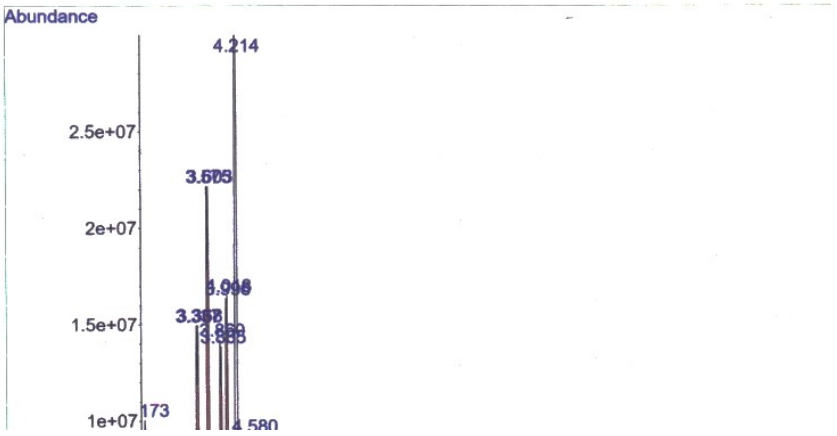

Figure 2. Representative GCMS chromatography data on B. coagulans bacterial filtrate

Table 1. MVCs identified in B. coagulans filtrates

Peak	Areas	Identified MVCs	Peak	Areas	Identified MVCs
1	7.61	hexanal;	15	0.55	ethylene glycol monohexyl ether;
2	2.05	cyclopentanol, 3-methyl;	16	5.17	isoheptane;
3	0.36	cyclopentanone, 3-methyl	17	4.71	isoheptane;
4	3.07	propanoic acid, 2-hydroxy	18	5.19	sulfurous acid, isohexyl 2-propyl
5	0.47	benzene, 1,4-dimethyl;	19	5.79	sulfurous acid, isohexyl 2-propyl
6	0.41	acetic acid, anhydride	20	23.5	oxalic acid, dodecyl ester
7	0.34	ethanol, 2-2-aminoethoxy;	21	4.43	tryptene
8	5.98	acetaldehyde, dihexyl acetate;	22	4.32	oxalic acid, cyclohexyl tetradecyl
9	5.86	1-hexyl iodide;	23	1.57	oxalic acid, cyclohexyl tetradecyl
10	0.15	propanal, dimethylhydrazone;	24	0.33	allyl acetone;
11	0.27	urea, 1,1-dimethyl;	25	0.18	1-cathinone;
12	7.97	pentane, 2,3,3,4-tetramethyl;	26	0.27	1H-indole-3-ethanamine;
13	8.71	3-1,3-dimethylbutoxy-2-butanol;	27	0.02	decamethyltetrasiloxane;
14	0.46	cis-2-buteneoxide;	28	0.26	decamethyltetrasiloxane;

The results of GCMS analysis of strawberry leaf extract in the control treatment showed that there were 24 peaks indicating the presence of 24 compounds contained in the strawberry leaf extract in the control treatment (Figure 3). Twenty-four of these compounds are methyl n-butyl ketone; n-nitroso-n-methylurea; trans-2-hexenol; cyclopentanone, 3-methyl; n-hexylacetamide; 2-hexanone, 3,3-dimethyl; 2-propenoic acid, methyl ester; 2-propenoic acid, methyl ester; isohexadecane; (3-aminopropyl) methylamine; stearaldehyde; 1-octadecene; ethyl 1-cyclohexyl ketone; 2,3-dimethyl-2-pentene; 2-hexadecen-1-ol; 1-tetradecene; neophytadiene; 2-acetylbenzoic acid; neophytadiene; 2,4-heptadienal; d-mannitol;butanedioicacid, dimethyl ester; palmitoyl chloride; and cyclopentadecanone, 2-hydroxy- (Table 2).

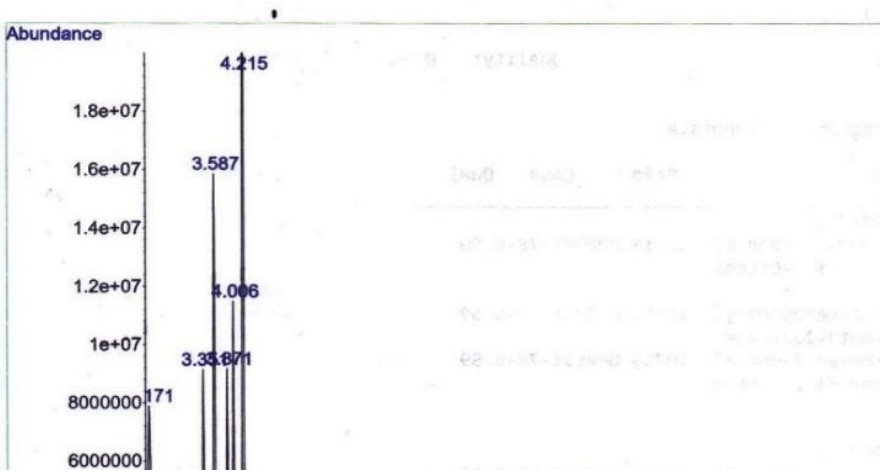

Figure 3. Representative GCMS chromatography data of strawberry leaf extract control treatment

Table 2. Compounds identified in strawberry leaves in the control treatment

Peak	Areas	Identified compounds	Peak	Areas	Identified compounds
1	11.64	methyl n-butyl ketone	13	24.80	ethyl 1-cyclohexyl ketone
2	0.36	n-nitroso-n-methylurea	14	3.16	2,3-dimethyl-2-pentene
3	1.88	trans-2-hexenol	15	2.59	2-hexadecen-1-ol
4	0.76	cyclopentanone, 3-methyl	16	0.94	1-tetradecene
5	0.41	n-hexylacetamide	17	0.28	neophytadiene
6	8.66	2-hexanone, 3,3-dimethyl	18	0.22	2-acetylbenzoic acid
7	0.33	2-propenoic acid, methyl ester	19	0.17	neophytadiene
8	14.50	2-propenoic acid, methyl ester	20	1.08	2,4-heptadienal
9	0.36	isohexadecane	21	7.55	d-mannitol
10	0.66	(3-aminopropyl) methylamine	22	0.24	butanedioicacid, dimethyl ester
11	8.04	stearaldehyde	23	1.45	palmitoyl chloride
12	9.50	1-octadecene	24	0.42	cyclopentadecanone, 2-hydroxy-

The results of GCMS analysis of strawberry leaf extract in the B. coagulans treatment showed that there were 40 peaks indicating the presence of 40 compounds contained in the strawberry leaf extract in the B. coagulans treatment (Figure 4). Forty of these compounds are 2-butyl glycol acetate; 1-tetradecene; octadecane; 2-norbornanol; cytosine riboside; 2,5-difluorobenzoic acid, 5-pentadecyl ester; succinic imide; 1-hexadecene; isohexadecane; (3-aminopropyl) methylamine; stearaldehyde; 1-octadecene; octadecane; 2-methyl-1-hexadecanol; 2-hexadecen-1-ol; 1-tetradecene; neophytadiene; 2-acetylbenzoic acid; neophytadiene; 2,4-heptadienal; 2-octylfuran; palmitic acid; pentadecyl heptafluorobutyrate; linolenic acid; hexadeca-2-, 14-diyne; cyclohexene; 7-heptadecyne; and1-naphthalene-sulfonic acid (Table 3).

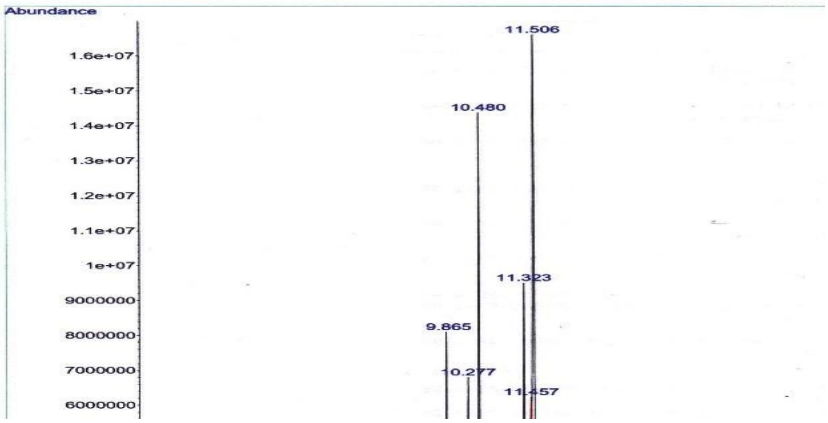

Figure 4. Representative GCMS chromatography data of strawberry leaf extract in treatment B. *coagulans*

Table 1. Compounds identified in strawberry leaves in the B. coagulans treatment

Peak	Areas	Identified compounds	Peak	Areas	Identified compounds
1	0.79	2-butyl glycol acetate	21	6.21	2-octylfuran
2	0.27	1-tetradecene	22	2.56	palmitic acid
3	0.58	octadecane	23	13.34	palmitic acid
4	0.55	2-norbornanol	24	0.13	pentadecyl heptafluorobutyrate
5	1.53	cytosine riboside	25	0.33	linolenic acid
6	0.47	2,5-difluorobenzoic acid,5-pentadecyl ester	26	7.44	linolenic acid
7	0.23	succinic imide	27	5,11	linolenic acid
8	0.32	1-hexadecene	28	28.93	linolenic acid
9	0.59	isohexadecane	29	2.33	hexadeca-2-, 14-diyne
10	0.96	(3-aminopropyl) methylamine	30	5.14	cyclohexene
11	0.40	stearaldehyde	31	0.55	7-heptadecyne
12	0.45	1-octadecene	32	0.46	1-naphthalene-sulfonic acid
13	0.25	octadecane	33	0.46	1-naphthalene-sulfonic acid
14	0.24	2-methyl-1-hexadecanol	34	1.60	1-naphthalene-sulfonic acid
15	4.78	2-hexadecen-1-ol	35	0.82	1-naphthalene-sulfonic acid
16	0.77	1-tetradecene	36	0.41	1-naphthalene-sulfonic acid
17	0.84	neophytadiene	37	0.70	1-naphthalene-sulfonic acid
18	0.16	2-acetylbenzoic acid	38	0.71	1-naphthalene-sulfonic acid
19	1.24	neophytadiene	39	0.11	1-naphthalene-sulfonic acid
20	1.67	2,4-heptadienal	40	1.19	1-naphthalene-sulfonic acid

Based on the literature and analysis of GCMS results, it shows that there are no compounds that have antiviral activity from the 24 compounds identified in strawberry leaf extract in the control treatment. Meanwhile, based on literature and analysis of GCMS results of strawberry leaf extract in B. coagulans treatment, it shows that there are 4 compounds that have antiviral activity out of the 40 compounds identified. The four antiviral compounds are cytosine riboside, palmitic acid, linolenic acid, and 1-naphtalene-sulfonic acid.

The results of this study show that MVCs produced by B. coagulans are able to induce the formation of 4 antiviral compounds in strawberry plants. The mechanism of MVCs in inducing resistance in strawberry plants to SVBV infection is not yet fully understood, however this research provides new information that 4 compounds have been known to have antiviral activity against viruses that infect humans and animals, but in this study 4 antiviral compounds were formed in the leaves of strawberry plants which was induced by MVCs produced by B. coagulans and was proven to be able to reduce symptoms and accumulation of SVBV in strawberry plants.

The cytosine riboside compound is a synonym for the cytidine compound which has antiviral activity against HIV-1 provirus (Ajoge et al., 2023), covid-19 (Burke et al., 2022), and Trichomonasvirus (Narayanasamy et al.,

2022). The mechanism by which the cytosine riboside compound inhibits viral replication is by inhibiting the formation of nucleic acids through a nucleotide analog mechanism. Palmitic acid and linolenic acid are fatty acid compounds. According to Perez et al. (2019) that fatty acid compounds can increase mammalian immunity against viral infections by increasing cytokine production in macrophages, inducing apoptosis, and regulating autophagy in hepatocytes. The 1-naphthalene-sulfonic acid compound has antiviral activity against HIV type 1 (Rusconi et al., 1996; Wang et al., 2004), Norovirus (Tarantino et al., 2014), Vesicular Stomatitis Virus (Bonafe et al., 2000), Respiratory syncytial virus (RSV) and Influenza A virus (Ikeda et al., 1994). The mechanism of the 1-naphtalene-sulfonic acid compound in inhibiting virus replication is by inhibiting the enzymes DNA dependent DNA-polymerase, ribonuclease, RNA-dependent RNA polymerase, and reverse transcriptase (Ilina et al., 2012; Wang et al., 2004; Tarantino et al., 2014).

Currently there are no published research results regarding the use of MVCs produced by B. coagolans to induce the formation of the antiviral compound cytosine riboside (1.53%); palmitic acid (15.9%); linolenic acid (41.81%); and 1-naphthalene-sulfonic acid (6.46%) in strawberry plants. These four antiviral compounds are thought to contribute to increasing the resistance of strawberry plants to SVBV infection.

4. Conclusion

B. coagulans MVCs are able to reduce symptoms caused by SVBV infection and reduce virus accumulation in plants based on NAE. Twenty-eight MVCs produced by B. coagulans were able to induce the formation of 4 antiviral compounds, namely cytosine riboside; palmitic acid; linolenic acid; and 1-naphthalene-sulfonic acid in strawberry plants.

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