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Full Length Article

Systemic Resistance Induced by *Stenotrophomonas maltophilia* Sg3 against Cucumber Mosaic Virus in Tobacco Plant

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Abstract

Cucumber mosaic virus (CMV) is one of the plant viruses that greatly interfere with the growth, quantity and quality of tobacco leaves. It is difficult to control the CMV, since it has a high genetic diversity, wide host range, and transmitted by insect vector. It is necessary to develop an alternative control method through induced systemic resistance (ISR) using rhizobacteria. This study was done to evaluate the potential of a rhizobacterium, *Stenotrophomonas maltophilia* Sg3 to induce antiviral activity against CMV in tobacco plant. Presences of CMV in the tobacco plant was detected based on double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) method, while the detection of compounds produced in the leaves of tobacco was done using gas chromatography mass spectrophotometer (GC-MS). Results of this study showed that treatment with *S. maltophilia* Sg3 significantly ($P < 0.05$) reduced the accumulation of CMV virus in the leaves of tobacco when compared to untreated leaves. In addition, this treatment significantly increased the leaves wet and dry weight, plant height, number of leaves per plant, and leaves chlorophyll content when compared to the leaves of control. These results suggested that *S. maltophilia* Sg3 was able to suppress the accumulation of CMV in the leaves, suppressed the symptom resulted from CMV infection as well as promoted the growth of tobacco plant. A compound, 2-naphthalene-sulfonic acid was detected in the leaves of plants treated with *S. maltophilia* Sg3, while no such compound was detected in the leaves of control plant. This compound might be responsible for the antiviral activity against CMV in tobacco. Results of this study suggested that *S. maltophilia* Sg3 can be considered as one of potential bio-agents for inducing antiviral activity against CMV in tobacco and promoted the growth of tobacco plant © 2020 Friends Science Publishers

Keywords: Induced systemic resistance; *Stenotrophomonas maltophilia*; Cucumber mosaic virus; Tobacco

Introduction

Cucumber Mosaic Virus (CMV) is one of important plant viruses on tobacco that may cause considerable economic losses, because, in addition to reducing production, it also greatly affects the quality of leaves produced (Qiu *et al*. 2018). Tobacco leaves infected with CMV generally shows several types of symptoms such as mosaic, chlorosis, and rolling (Gal-on *et al*. 1996; Qiu *et al*. 2018). To date, control of disease caused by CMV is still very difficult, due to a high genetic diversity of CMV (Schneider and Roossinck 2001), wide range of CMV host plants, and CMV can be transmitted by various types of aphids non-persistently (Pinto *et al*. 2008). Until now there is no chemical compound that can specifically control the development of the virus without affecting its host plants. Efforts should be made to find a bio-agent that in one hand can suppress the multiplication of virus in its host plant, and on the other hand it does not interfere with the plant growth and development.

One of the plant virus control technologies is Induced Systemic Resistance (ISR) technology which is induced by rhizobacteria. Ramamoorthy *et al.* (2001) reported that ISR is a plant resistance that occurs due to biochemical changes or plant physiology which then stimulates the formation of PR (pathogenesis related) proteins, synthesis of phytoalexin, and other secondary metabolites. Some researchers reported that rhizobacteria can induce the systemic resistance in plants through inhibition of viral replication-related proteins, activation of plant resistance genes, formation of enzymes, and secondary metabolites. Li *et al.* (2016) reported that treatment of cucumber plants with *Stenotrophomonas maltophilia* HW2 was able to reduce the development of symptoms caused by *Cucumber Green Mottle Mosaic Virus* infection. *S. maltophilia* HW2 increased the resistance of cucumber plants by inhibiting gene expression of replication-related proteins such as CP, MP, and Rep that inhibiting viral replication in the leaves and rapidly inducing

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the expression of plant resistance genes such as PR1 and PR5. Similar result was shown by Borollosy and Oraby (2012) that treatment of *Azotobacter chroococcum* into cucumber plants was able to reduce the development of symptoms caused by CMV infection and reduced the accumulation of virus in plants by inducing peroxidase enzymes and ß-1, 3-glucanase. Resistance against viral infection was also reported by Beris *et al*. (2018) that treatment of tomato plants with *Bacillus amyloliquefaciens* strain MBI600 increased the resistance of tomato plants against Tomato Spotted Wilt Virus and Potato Y virus infection by inducing the formation of salicylic acid compounds. Other study was done by Park *et al*. (2008) that *Pseudomonas chlororaphis* O6 increased the resistance of tobacco plants against pathogenic bacterium *P. syringae* pv. *tabaci* infection by inducing the formation of 4- (amino carbonyl) phenyl acetate compounds. Treatment with *P. aeruginosa* PM12 was reported to increase the resistance of tomato plants to *Fusarium oxysporum* infection by inducing the formation of 3-hydroxy-5-methoxy benzene methanol (HMB), eugenol and tyrosine compounds (Fatima and Anjum 2017).

S. maltophilia Sg3 was isolated from rhizosphere of *Arachis hyphogea* and has been proven to be effectively promoted the growth and increased the yield of Edamame soybean (Khamdan *et al*. 2017). In this study *S. maltophilia* Sg3 was tested for its capability to induce resistance against CMV in order to find an alternative bio-control agent to manage CMV infection in tobacco plant.

Materials and Methods

Bacterial culture and tobacco plant treatment

S. maltophilia Sg3 was isolated from rhizosphere of groundnut (*Arachis hyphogea*) and maintained in the Laboratory of Biopesticide, Faculty of Agriculture Udayana University, Bali Indonesia. The bacterium was grown in *Stenotrophomonas* selective agar base medium (10 g peptone, 10 g mannitol, 0.06 g bromothymol blue, 20 g agar, and distilled water in one liter) for 48 h and incubated at 35°C. The bacterial colony was harvested using sterile distilled water and the density was adjusted to 10^8CFU/mL. Fifteen tobacco plants each for treatment, and control were used in this experiment. Fifty milliliter of *S. maltophilia* Sg3 suspension at a density of 2×10^8 CFU was poured onto tobacco plants aged a month at soil surface close to plant basal. As for the control, tobacco plants were dressed with 50 mL of sterile distilled water. Seven days after treatment, tobacco plants of control as well as the plants treated with *S. maltophilia* Sg3 were inoculated with CMV mechanically. All of tobacco plants were grown and maintained in a green house.

Inoculums and inoculation

Inoculums as the source of CMV was obtained from the leaves of tobacco infected with CMV that maintained in a freezer (-20°C). The infected leaves were put in a mortal and `homogenized with a pestle and then added with 0.01 *M* phosphate buffer saline (PBS) pH 7.0. One gram of infected leaves was added with 5 mL of PBS. The suspension obtained from this procedure was used as inoculums. The inoculums were inoculated into two fully developed youngest leaves. The leaves were scattered with carborundum on the upper surface, and then inoculated with inoculums. Inoculums were rubbed with sterile cotton starting from the bottom to the top of the leaves in the same direction. About 5 min after inoculation, the inoculated leaves were sprayed with sterile distilled water to remove the carborundum on the leaves.

Detection of CMV and determination of leaf chlorophyll levels

Virus detection was carried out at 2 weeks after inoculation. Detection was conducted according to the DAS-ELISA serological method using CMV antiserum (Agdia). DAS-ELISA results were analyzed quantitatively by ELISA reader (Bio-RAD 550-microplate reader model, Tokyo, Japan) at a wavelength of 405 nm. The result was considered positive if the absorbance value of ELISA was 2 times higher than that of the negative control (healthy plant). One gram of leaves respectively for CMV-infected young leaves, and healthy leaves were homogenized on a mortar and 10 mL PBS (10 m*M* Na_2HPO_4, 0.1 *M* NaCl, pH = 7.0). Homogenate was filtered with filter paper and the filtrate was collected. The ELISA plate wells were coated with anti-CMV immunoglobin g (IgG) at 1.5 μg/mL in the buffer layer (35 m*M* Na_2HCO_3, 15 m*M* $Na2CO_3$, 0.2% bovine serum albumin (BSA), and 2% polyvinyl pyrolidone, pH 9.6,100 μL / well), and incubated at 4°C for 12 h. Plates were washed three times with PBS containing 0.05% Tween-20. The well was then loaded with leaf extract (100 μL / well) and incubated at 4°C for 12 h and washed three times as before. IgG conjugated with alkaline phosphatase at 1: 2000 dilution in conjugate buffer was added (100 μL / well). Plates were incubated at 37°C for an hour. The plates were washed as described previously and the substrate P-nitro phenyl phosphate at 1 mg/mL in 10% diethyl amine pH 9.8 was added (100 μL / well) and the absorbance value was measured at 405 nm using ELISA reader (Bio-RAD 550-microplate reader model, Tokyo, Japan).

Since CMV infection resulted in the change in chlorophyll content, the level of chlorophyll in the leaves of tobacco was determined using Chlorophyll-meter SPAD-502 (Konica Minolta, Japan).

Detection of antiviral compounds in the leaves

Detection of antiviral compounds in the leaves of tobacco was carried out 15 days after inoculation of CMV. Ten grams of tobacco leaves were put in a mortar and added

with liquid nitrogen and crushed with pestle until fine and dissolved in 100 mL of an extraction solution consisting of methanol and ethyl acetate in a ratio of 1:1. After 48 h, the solution was evaporated using a vacuum rotary evaporator at 40°C. The extract was then partitioned using methanol and hexane with a ratio of 1:1 in a separating funnel. Methanol and hexane phase solutions were evaporated using a vacuum rotary evaporator at 40°C. Furthermore, detection of antiviral compounds was carried out using Gas Chromatography-Mass Spectrometer (GC-MS QP2010 Ultra, Shimadzu).

Results

Results of this study showed that treatment of tobacco plants with *S. maltophilia* Sg3 prior to inoculation with CMV effectively suppressed the CMV accumulation in the leaves as well as reduced the symptom resulted from CMV infection. Treatment with *S. maltophilia* Sg3 was able to reduce the accumulation of CMV in plants by 74%. This was determined based on the value of ELISA absorbance at 405 nm where the average value for control plants was 2.863 while for treated plants was only 0.168 (Table 1). The symptom of CMV infection was severe on plants of control with mosaic and dwarf symptom, while no such symptom was observed on treated plants (Fig. 1). The wet and dry weight of leaves of treated plants were significantly ($P < 0.05$) higher than that of control. The differences were 51 and 110%, respectively for wet and dry weight of leaves. The plant height and number of leaves per plant were also significantly ($P < 0.05$) higher on treated plants when compared to control, with differences by 86 and 31% respectively for plant height and number of leaves per plant (Table 1). The level of chlorophyll in the leaves of plants treated with *S. maltophilia* Sg3 was also significantly ($P < 0.05$) higher on control plants, with the difference by 38%.

Result of GC-MS analysis showed that 8 compounds were detected in the leaves of tobacco plants treated with *S. maltophilia* Sg3 (Fig. 2A and Table 2) while only 4 compounds were detected in the leaves of plants of control (Fig 2B and Table 3). Among 8 compounds detected in the leaves of treated plants, 2-Naphthalene-sulfonic acid has been known to possess antiviral activity. This compound was not detected in the leaves of control plants.

Discussion

Treatment with *S. maltophilia* Sg3 on tobacco plants prior to inoculation with CMV was proven to be effectively suppressed the accumulation of CMV in the leaves as well as suppressed the development of CMV infection symptom in tobacco plants. The plants of control showed mosaic and severe dwarf symptom while no such symptom was observed on the plants treated with *S. maltophilia* Sg3. These results suggested that treatment with *S. maltophilia* Sg3 induced systemic resistance in tobacco plants against CMV infection. Based on the data of the growth parameters can be considered that *S. maltophilia* Sg3 was also act as growth promoter.

Several previous studies reported that treatment with bio-agents successfully induced resistance against CMV on other plant (Galal 2006; Al-ani and Adhab 2012; Latake and Borkar 2017). Al-Ani and Adhab (2012) reported that seed treatment and dressing with *P. fluorescens* suspension was able to reduce CMV accumulation in melon plants. Other report by Galal (2006) showed that cucumber seeds soaked in a filtrate of *Actinomycetes* for 2 h effectively reduced CMV symptoms. Cucumber seeds soaked in a filtrate of *Streptomyces olivaceus* for 8 h showed a less CMV symptom when compared to control (Latake and Borkar 2017). According to Walters *et al.* (2013) treatment of plants with biotic and non-biotic agents can induce systemic resistance to plant pathogens, both locally and systemically. Induced resistance is characterized by restrictions on pathogen growth and suppression of the development of symptoms of the disease.

The mechanism of *S. maltophilia* Sg3 in reducing the accumulation of virus in tobacco plants and reduce the symptom of CMV is probably through the production of antiviral compounds that induced systemic resistance against CMV. Result of GC-MS analysis showed that the plants treated with *S. maltophilia* Sg3 produced 2-Naphthalene-sulfonic acid, while no such compound was detected in the leaves of control plants. This 2-Naphthalene-sulfonic acid has been reported to posses antiviral activity. Hiyama (1952) reported that hydroxyl naphthalene sulfonic acid derivative compounds such as 1-naphthol 2- (3-, 4-, 5-) sulfonic acid; 1-naphthol-3, 6- (3, 8-) disulfonic acid, 1- (2-) naphthol-3, 6, 8 trisulfonic acid; 2-naphthol-l- (6-,7-) sulfonic acid; 1 2-dihydroxynaphthalene-4-sulfonic acid; 1,2-naphthoquinone-4-sulfonic acid; 2-amino1-naphthol-4-sulfonic acid; l-naphthol-4-sulfonamide; l-ethoxynaphthalene-4-sulfonamide and N-(3-hydroxy-4-oxo-l-naphthylidene)-naphthionic acid have antibacterial and antiviral activity. Compound 2-naphthalene-sulfonic acid is a direct parent compound of naphthalene sulphonate which has antiviral activity. Wang *et al.* (2004) proved that 2-naphthalene sulfonic acid (4-hydroxy-7- [[[5-hydroxy-6 -[(4cinnamylphenyl) AZO] -7-sulfo-2-naphthalenyl] amino]-carbonil] amino] -3 [(4cinnamylphenyl) AZO (KM-1)) inhibited the enzyme non-nucleoside reverse transcriptase in type 1 HIV virus. The reverse transcriptase enzyme in type 1 HIV virus is an enzyme that catalyzes the replication of single-stranded RNA into double-stranded DNA that occurs during the viral infection process in host cells and has functions such as DNA-polymerase dependent DNA and ribonuclease H (Ilina *et al*. 2012). Other researchers reported that Naphthalene sulfonate inhibits type 1 HIV replication in lymphoblastoid cells (Rusconi *et al*. 1996). Similar report was published by Tarantino *et al.* (2014) that Naphthalene sulfonate inhibits the RNA-dependent RNA polymerase enzyme in Norovirus, a virus that causes

Table 1: Performance of tobacco plants inoculated with CMV with and without application of *S. maltophilia* Sg3

Treatment	ELISA Absorbance value	Leaf wet weight (gram)	Leaf dry weight (gram)	Plant height (cm)	Number of leaves	Leaf chlorophil level (SPAD unit)
Control (inoculated with CMV only)	2.863 a*	212.7 a	17.7 a	35 a	13 a	32.98 a
Treatment with *S. maltophilia,*and inoculated with CMV	0.168 b (-74%)**	321.6 b (+51%)	37.3 b (+110%)	65 b (+86%)	17 b (+31%)	45.64 b (+38%)

*The values followed by the same letter in the same column shows non-significant differences ($P > 0.05$) according to T-test at 5% level
**Values in the parenthesis indicate percentage of decrease (-) and percentage of increase (+) when compared to tobacco plant without treatment of *S. maltophilia* Sg3

Table 2: Compounds detected in the leaves of tobacco (treated with *S. maltophilia* Sg3)

Peak	Retention time	Compound identified	Area (%)	Molecule weight (g/mol)	Molecule formula
1	6.73	2-Pentanone, 4-methoxy-4-methyl-	2.55	130,187	$C_7H_{14}O_2$
2	11.41	1-ISOPROPYL-4-METHYL-3-CYCLOHEXEN-1-OL	4.97	198,306	$C_{12}H_{22}O_2$
3	11.70	Beta-Phellandrene	16.82	136,238	$C_{10}H_{16}$
4	14.24	Nicotine	46.51	162,236	$C_{10}H_{14}N_2$
5	16.39	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(.alpha., 7.alpha., 8a.beta.)]	4.40	204,3511	$C_{15}H_{24}$
6	16.49	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(.alpha., 7.alpha., 8a.beta.)]	3.09	204,3511	$C_{15}H_{24}$
7	25.21	2-Naphthalene-sulfonic acid	16.94	226,246	$C_{10}H_8O_3S$
8	28.34	Diethyl-1-(carb-n-butoxy)propylphosphonate	4.71	280,301	$C_{12}H_{25}O_5P$

diarrheal disease in humans. Bonafe *et al.* (2000) proved that the Bis-(8-anilinonaphthalene-1-sulfonate) compound was able to incentivize vesicular stomatitis virus, while Ikeda *et al.* (1994) reported that polymer sulfonic acid was able to inhibit the replication of respiratory syncytial virus (RSV) and influenza virus A.

Until now there is very little information about the use of bio-agents to induce the formation of 2-Naphthalene-sulfonic acid compounds in plants. The results of this study indicate that the *S. maltophilia* Sg3 induced the formation of 2-Naphthalene-sulfonic acid compounds in tobacco plants so as to reduce the accumulation of viruses in plants as well as reduced the symptoms caused by CMV infection. The mechanism of *S. maltophilia* Sg3 induces the formation of 2-Naphthalene-sulfonic acid compounds in tobacco plants is not yet fully understood, but this study provides interesting information that so far 2-naphthalene-sulfonic acid has been known to have antiviral activity against viruses that infect humans and animals. In this study 2-Naphthalene-sulfonic acid formed in the leaves of tobacco plants induced by *S. maltophilia* Sg3 can be considered as one of mechanisms by which induced systemic resistance against CMV infection was performed.

There are several bio-agents and compounds that have been reported to have antiviral activity against tobacco mosaic virus (TMV). Li *et al.* (2008) reported that *S. noursei* var. *xichangensis* was able to induce resistance in tobacco plants against TMV by producing ningnanmycin compound. This compound was able to destroy the TMV coat protein. Report by Naidu *et al.* (2012) stated that the synthetic of 14-Aryl / Heteroaryl-14H-dibenzo [a, j] Xanthene has antiviral activity against TMV. Synthesis of this compound was done using polyethylene glycol sulphonic acid (PEG-OSO3H) catalyst. Bitriazolyl acyclonucleosides was reported has antiviral activity against TMV (Li *et al.* 2008). Fan *et al.* (2011) reported that spraying ofO, O-diisopropyl (3- (L-1-(benzyl amino)-1-oxo-

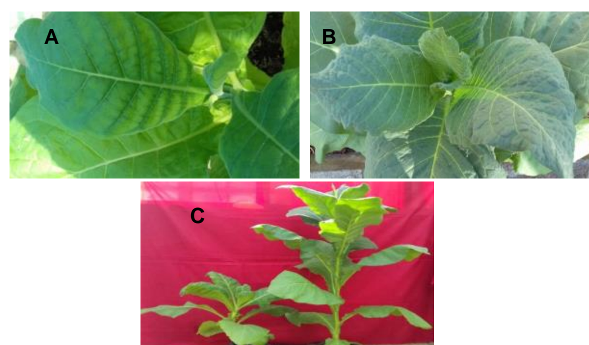

Fig. 1: Symptoms of tobacco plants with and without treatment of *S. maltophilia* Sg3
A. tobacco plant of control that showed mosaic symptom, B. tobacco plants treated with *S. maltophilia* Sg3 without mosaic symptom, C. tobacco plant of control that showed dwarf symptom (left), and tobacco plant treated with *S. maltophilia* Sg3 without dwarf symptom (right)

3-phenylpropan-2-yl) thourourido (phenyl)-methylphosphonate on tobacco leaves was able to inhibit the TMV coat protein polymerization process *in vitro*. In our present study we confirmed that systemic resistance against CVM in tobacco plant by *S. maltophilia* Sg3 was the most probably through the production of secondary metabolite. Further study is needed to explain the mechanism of 2-Naphthalene-sulfonic acid in suppressing CMV replication and infection. It is also important to know whether 2-Naphthalene-sulfonic acid is solely responsible for induced systemic resistance against CMV in tobacco or work together with other compounds

Conclusion

Treatment with *S. maltophilia* Sg3 effectively reduced the accumulation of CMV in the leaves of tobacco and suppressed the development of CMV symptom. This bacterium also promoted the growth of tobacco plant

Table 3: Compounds detected in tobacco leaves (control plants)

Peak	retention time	Compound identified	Area (%)	Molecule weight (g/mol)	Molecule formula
1	11.70	4-Aminothiophenol	8.55	125,189	C_6H_7NS
2	14.25	Nicotine	10.75	162,236	$C_{10}H_{14}N_2$
3	25.21	Cis/trans-spiro[1,2-Dihydro-1-oxoacenaphthylene-2,7'-8'-oxabicyclo[4,2.0]octane]	12.42	342,489	$C_9H_{27}AsO_3Si_3$
4	26.18	2-Methyl-5H-dibenz[b,f]azepine	68.28	207,270	C15H13N

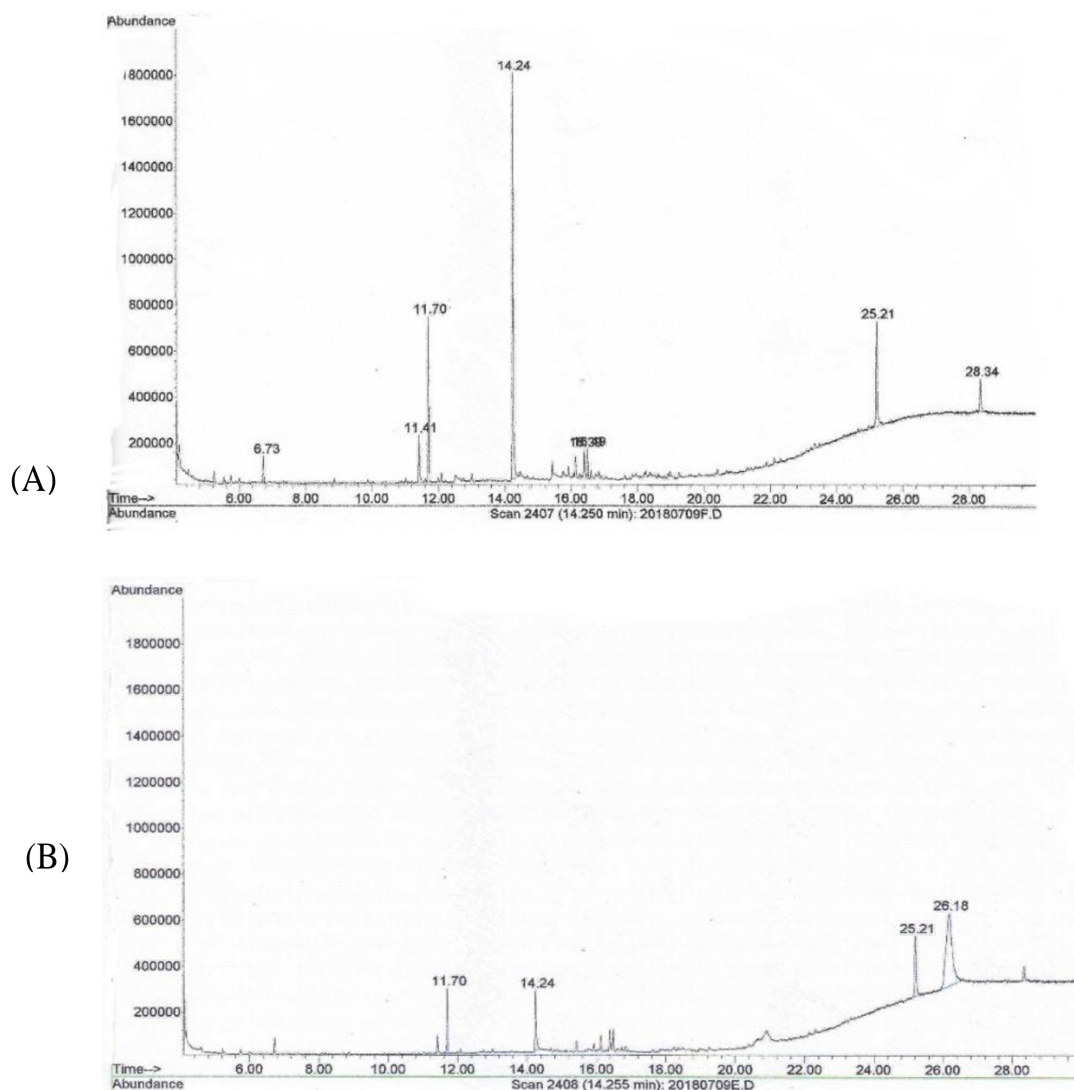

Fig. 2: Chromatograms of compounds in the leaf extracts of tobacco based on GC-MS analysis. (**A**) compounds detected in tobacco leaf extract treated with *S. maltophilia* Sg3; (**B**) compounds detected in tobacco leaf extract of control

indicated by the higher wet and dry weight of leaves, plant height, number of leaves per plant, and leaves chlorophyll level. A compound, 2-naphthalene-sulfonic acid was detected in the leaves of plants treated with *S. maltophilia* Sg3, which might be responsible for the antiviral activity against CMV in tobacco. This study proved that *S. maltophilia* Sg3 can be considered as one of potential bio-agents to be further developed to induce systemic resistance against CMV in tobacco and promoted the growth of tobacco plant.

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