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Induction of Salicylic Acid in Cucumber against ZYMV *Potyvirus* by some Nutrient Chemicals

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Systemic acquired resistance (SAR) could be induced in cucumber plants using different concentrations of seven nutrient chemicals against *Zucchini yellow mosaic Potyvirus* (ZYMV). These chemicals were, Potassium sulfate, Magnesium sulfate, Ammonium sulfate, Chelated iron, Chelated zinc, Chelated manganese, and salicylic acid. SAR was determined via disease severity, virus concentration and some biochemical changes i.e., the high level of endogenous salicylic acid, proteins related to inducers and activity of peroxidase and chitinase enzymes. Salicylic acid (0.5%) and potassium sulfate (3%) gave the highest effect in inducing SAR, where the treatment with them had forbid disease symptoms, and virus concentration was (0), 25 days post inoculation. In addition the biochemical changes reached to the maximum values, whereas other chemicals gave a medium ability to induce SAR and gave varied values.

INTRODUCTION

The phenomenon of systemic acquired resistance (SAR), refers to a distinct signal transduction pathway which can make plants to be stimulated to defend themselves against pathogens. SAR activation is a broad, physiological immunity results from infection with necrotrophic pathogens, and also, certain natural and systemic chemical compounds can trigger similar plant responses (Neuenschwander *et al.*, 1996).

There is a number of biochemical and physiological changes have been established to be associated with SAR state. These include, cell death and the oxidative burst (Low and Merida, 1996), deposition of callose and lignin (Kauss, 1987), the synthesis of phytoalexins (Dixon, 1986) and proteins (Neuenschwander *et al.*, 1996 and Sabry, 2003). Many of novel proteins associated with SAR, belong to the class of pathogenesis related (PR) proteins, these proteins are classified as SAR proteins, when its presence and activity correlates tightly with maintenance of the resistance state. The induced proteins in a particular plant species have co-evolved to be somewhat specific for plant species and its pathogens (Neuenschumder *et al.*, 1996). In cucumber, SAR is correlated with increasing in peroxidase, and class III chitinase activity, both which considered as a SAR marker in cucumber (Métraux and Boller, 1986 and Smith and Hammerschmidt, 1988)

SA was considered as an important component in the signal transduction pathway leading to SAR to the entire spectrum of plant pathogens: bacteria, fungi and viruses.

Malamy *et al.* (1990) and Métraux *et al.* (1990) showed that the increase of SA by several hundred fold and the appearance of SA in phloem sap and in upper non infected leaves of cucumber, tobacco and Arabidopsis are correlated with the onset of SAR

The use of chemical inducers eliminates potential problems associated with the introduction of plant pathogens as inducers. Chemicals are more readily produced, distributed, and stored than are pathogen. Also chemical inducers could be applied

with the techniques and equipment of mechanized agriculture to minimizing labor and express involved in resistance induction. (Kessmann *et al.*, 1994)

Kessmann *et al.* (1994) illustrate that to be considered an activator of SAR, a chemical should exhibit three characteristics, first, the compound or its significant metabolites should not exhibit direct antimicrobial activity, second it should induce resistance against the same spectrum of pathogens as in biologically activated SAR, and third it should induce the expression of the same marker genes as evident in pathogen-activated SAR. There are many chemical inducers have been reported include: oxalate (Doubrava *et al.*, 1988) di and tribasic phosphates (Gottstein and Kuc, 1989) B-ionone (Salt *et al.*, 1986), 2,6-dichloroisonicotinic acid and its methylester derivative (Métaux *et al.*, 1991), Fosethyl-Al, metalaxyl (Ward, 1984), triazoles (Hauthal, 1993), 2,2-dichloro-3.3-dimethyl cyclopropane carboxylic acid (DCP) (Langcake and Wickins, 1975), probenazole (Kato *et al.*, 1984), Jasmonic acid (JA) (Cohen *et al.*,1993), and others.

In this work, some of simple nutrient chemicals used in different concentrations to induce resistance in cucumber plants under greenhouse conditions to make use of this phenomenon in resistance of virus infection. Many indicator measurements associated with this phenomenon were performed.

MATERIALS AND METHODS

Chemical agents

The chemical nutrient agents used in these experiments were Salicylic acid (SA), Potassium sulfate (PS), Ammonium sulfate (AS), Magnesium sulfate (MS), Chelated iron (CI), Chelated manganese (CM), and Chelated zinc (CZ). To determine which concentrations of each chemical material, will be used, different concentrations was applied (4 conc.), and just two were selected (Table1), after count out the concentrations, which cause a great injury to the leaf (Phytotoxicity), and which too low to cause stress on the plant to inciting it to resist. 200 ml of each concentration were prepared, and to improve spread, two drops of Tween 80 were added. Chemical agents were supplied from central lab. For agriculture climate (PS, AS, MS, CI, CM, CZ, are commercial fertilizers, SA was bought from El-Gaumhooria company, produced by Fluka)

Table (1): Selected concentrations of each material used with cucumber plants.

	SA	PS	AS	MS	CI	CM	CZ
Conc %	0.25	2	0.25	2	0.25	0.5	0.5
	0.5	3	0.5	3	0.5	1.0	1.0

Host plant and virus isolate

Cucumber (*cucumis sativus* L. cv. Atlas), seeds were imported by El-bosaly Research Unit, Central Lab. for Agriculture Climate, and *C. amaranticolor* Caste & Reyn seeds were supplied from virology greenhouses, Fac. of Agric, Ain-Shams University.

ZYMV isolate was gently supplied from Dr. Khalid EL-Dougdoug, Prof. Of Virology, Department of Microbiology, Faculty of Agriculture, Ain Shams University.

SAR experiments

All plants were growing in plastic pots (4 inch Ø) containing 1:1 mixture of Canadian peatmoss and vermculite supplemented with nutrients under plastic greenhouse. Plants were divided into two divisions;

first, for virus infectivity experiments, consists of 15 groups (10 plants per group), each material has two groups for two concentrations, as well one group for control. Second, for biochemical changes measurement, consists of 8 groups (10 plants per group) each group for one concentration from each material, which more effective in reducing virus infectivity, and one group for control.

Plants were sprayed using mist sprayer, at the first leaf stage and when second leaf stage began to grow. Whole plant was sprayed, especially on lower surface where stomata abundantly exist. After spraying, plants were shake by hand to remove suspended drops at the edges of leaf. Control plants were sprayed with water.

ZYMV was used in the challenge infection 7 days after spraying; all leaves of cucumber plant were inoculated. The inoculum was prepared using infected leaves, grounded in a mortar containing 0.1 M phosphate buffer pH 6.0, 1:2 (W/V), the homogenate was filtered through tow layers of cheesecloth. Leaves were slightly dusted with carborandum, (600 mesh) and rubbed gently with finger previously dipped into the virus preparation.

Virus infectivity in induced treated plants

Severity of Symptom

The symptoms on induced cucumber plants were examined 25 days post inoculation, using the following rating scale:

0 = no symptoms; 2 = chlorotic local lesions and mild mosaic; 4 = sever mosaic; 6 = blisters and malformation. Disease severity values were calculated using the following formula according to yang *et al.* (1996). Disease severity **(DS)** =

$$\frac{\text{(Disease grad} \times \text{number of plants in each grade)}}{\text{(Total number of plants) (Highest garde)}} \cdot 100$$

Measurement of Virus concentration in induced plants

In each group, virus concentration was measured in random sample consisted of 3 plants of *C. amaranticolor* Caste & Reyn using latin square method. *C. amaranticolor* plants were inoculated with 50 µl of virus-infected cucumber preparation per leaf, using a spatula. The concentration was measured as a number of local lesions.

Measurement of some biochemical changes in induced plants

After 7 days post spraying, with the effective concentration in reducing virus infectivity, leaves from every group of sprayed cucumber plants were collected and mixed together, and 4g were taken for determination of biochemical changes, 1g for SA quantification, 1g for determination of proteins related chemical inducers, 1g for peroxidase, and 1g for chitinase activity measurements.

Extraction and quantification of total Salicylic acid (SA) in induced Plants

SA was measured in treatments which effective in reduction of infection percentage and severity of symptoms. Samples were prepared as described by Raskin *et al.* (1989) with one modification that total free and conjugated SA was measured directly using β-glucosidase enzyme. One gram of frozen tissue was ground in 3ml of 90% methanol and centrifuged at 6000 g for 15min. the pellet was back extracted with 3ml of 99.5% methanol and centrifuged. Methanol extracts was combined, centrifuged at 1500 to 2000g for 10min, and dried at 40°C under vacuum using Rotary evaporator (Heidolph.). Dried extracts were then resuspended in 3 ml of water at 80°C and an equal volume of 0.2 M sodium

acetate buffer (pH 4.5) containing 0.1mg/ ml β-glucosidase (22 units/mg, sigma) was added. This mixture was incubated at 37°C overnight. After digestion, mixtures were acidified to pH 1 to 1.5 with HCL. SA was extracted for quantification by high performance liquid chromatography (HPLC) into 2 volumes of cyclopentain/ ethylacetute / isopropanol (50:50:1). The organic extract was dried under nitrogen and analyzed by HPLC as described previously (Raskin *et al.*1989).

2- Determination of proteins related chemical inducers

Proteins of induced plants were determined using sodium dodicyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis under nondenaturing conditions was performed in 12.5% (W/V) acrylamide slab gel according to the method of (Laemmli, 1970). The slap gel was electrophoresed at 25 to 30mA constant current and 150 to 200 V for 3 to 4 h. The gel was fixed in 12% trichloroacetic acid for 1 h and stained with coomassie blue R-250. Moleculer weight was determined by SDS-PAGE with markers (7, 22, 30, 36, 52, 98, 119, 205 KDa) obtained from Agric. of Genetic Engineering Researche Institution (AGERI)

3- Enzyme activity:
Peroxidase activity

Peroxidese activity was measured as described by Reuveni *et al.* (1991), 1 g of leaf tissue were homogenized with a mortar and pestle in 1 ml cold 0.015 M phosphate buffer, (pH6.0). The homogenate was centrifuged at 10,000 g for 10 min at 4 °C and the activity of peroxsidase in the supernatant was determined by adding 10µl of enzyme extract to 3ml of assay solution, consisting of 15mM sodium phosphat buffer pH6.0, 1mM H_2O_2 and 0.14 mM 0-methoxyphenol (guaiacol). The increase in optical density at 470nm was recorded using Jenway 6405UV/Vis Spectrophotometer.

Chitinase activity

The substrate colloidal chitin was prepared from chitin powder according to the method described by Ried and Ogryd-Ziac (1981). Twenty-five grams of chitin was suspended in 250ml of 85% phosphoric acid (H_3PO_4) and stored at 45C for 24 h, then blended in 2 liter of distilled water using a Warning blender and the suspension was centrifuged. This washing procedure was repeated twice. The colloidal chitin suspension in the final wash was adjusted to the pH 7.0 with (1N) NaOH, separated by centrifugation and the pelleted colloidal chitin was stored at 4 °C.

Protein extract was obtained by homogenizing frozen cucumber tissues (1g fresh weight) at 45C in 5 ml of 100 mM sodium acetate buffer, pH 5.0 and 2 % (W/V) polyvinylpyrrolidone (PVP), Mol. Wt. 40000. The homogenate was centrifuged at 27,000 g for 30 min at 4°C and clarified supernatant was recovered. (Merodio *et al.*, 1998)

Chitinase activity was assayed using colloidal chitin as a substrate. A reaction mixture containing 1ml of extract and 1ml colloidal chitin (20g.l-1) in 50mM sodium acetate buffer at pH 6.8 is incubated in a water bath for 1h at 375C. After that, the mixture was centrifuged at 2500g for 20 min. the concentration of N-acetyl-glucoseamine (NAGA) in the supernatant is determine at 550 nm with a fluorescence spectrophotometer (Jenway 6405UV/Vis). The activity of

chitinase was measured as change of absorbance h-1 ml-1 of the extract.

RESULTS AND DISCUSSION

Cucumber is a widely studied models system for acquired resistance (Neuenschwander *et al.*, 1996). Many inducers include bacterial, fungal and viral nicrotrophic pathogens and many several chemicals have been studied with cucumber against many pathogens (Doubrava *et al.*, 1988; Gottstein and Kuc 1989; and Reuveni *et al.*, 1992). In this study, simple fertilizer chemicals were applied in easy way to facilitate using of which succeeded in induced resistance via farmer. Using of external SA in these experiments was as a positive control, because many workers improved its ability to induce resistance in many crops: in tobacco (ward *et al.*, 1991); in Arabidopsis sp (Uknes *et al.*, 1992), and in Cucumber (Lowton *et al.*, 1993).

Virus infectivity

Seven nutrient chemicals were applied with cucumber plants. Each chemical material has two concentrations, as described before. Challenge infection was carried out 7 days post spraying. The general note, that inoculated plants varied in the severity of the symptoms, and some treatments showed some delay in symptom elicitation. So, severity of symptoms was measured 25 days post inC°ulation.

Disease severity (DS)

Data in Table (2) show that, a clear differentiation in the levels of DS was found; all are less than the control. The most effective chemical materials in reducing severity and percentage of infection were SA and PS, where DS was 0 % with 0.5 % of SA and 3 % of PS, whereas it was 6.6 and 10% with SA (0.25%) and PS (2%) respectively. MS also conduced to very low percentage of DS, where gave 16.7% with concentration of 3 %. CI (0.5%) and CZ (1%) treatments were conduced to a closed low values, where gave 20 and 23.3%, respectively. The highest DS values were given by CM (0.5%) and AS (0.25%), where gave 76.6 and 66.7% respectively.

Table (2) Disease severity in treated Cucumber plants

Chemicals	Conc .%	% of infection	DS (%)*	Symptom elicitation (dpi**)
CL	-	100	100	13-15
SA	0.25	20	6.6	22
	0.5	0	0	0
PS	2	30	10	20
	3	0	0	0
MS	2	80	43.3	21
	3	30	16.7	19
AS	0.25	100	66.7	13
	0.5	80	43.3	13
CI	0.25	100	50	17
	0.5	60	20	17
CZ	0.5	60	60	16
	1.0	40	23.3	20
CM	0.5	100	76.6	17
	1.0	60	40	19

* DS = disease severity
** dpi = day post inC°ulation

Virus concentration in induced treated plants

Virus concentration was measured in treatments, which give high resistance and less percentage of severity of symptoms. It was found that the decrease in severity has a relationship with the concentration of the virus in induced treated plants. All plants ,which inoculated with treated cucumber leaves, produced local lesions less than inoculated with

control, and reached (0) with SA (0.5%) and PS (3%) (Table 3).

The decrease in disease severity and virus concentration in induced plants agreed with the study of Naylor *et al.* (1998) with tobacco, they found that the treatment with exogenous SA can induce resistance to viruses by two different mechanisms, either induce interference with replication in the directly inoculated tissues, (as in local lesion infection with TMV or PVX); or it can induces an inhibition of long–distance movement (as in systemic infection with CMV). First mechanism affecting directly on replication, but the other ones, affecting on the movement of virus in the phloem, and both reduce the virus particles concentration in inoculated and uninoculated tissues.

Measurement of some biochemical changes in induced plants

1-Extraction and quantification of total SA in induced treated plants:

The obtained results from quantification of endogenous salicylic acid in induced treated cucumber plants using HPLC, agreed with disease severity and virus concentration results. Where, exogenous SA (0.5%) treatment gave the highest level of endogenous SA, since gave 907.2 µg /1g fresh weight (fw), followed with PS (3%), where gave 252 µg /1g fw of SA level. Table (3) and figure (1) show the different levels of endogenous SA level. MS (3%), CI (0.5%) CZ (1%) gave 115.2, 144 and 144 µg /1g fw, respectively. While, CM (1%) and AS (0.5%) were relatively low, although the DS percentage was 40 and 43.3 % respectively. The control value was 2.09 µg /1g fw.

2-Protein related chemical inducers

Eleocrtophoretic patterns of proteins and their molecular weights (MW) in treated cucumber plants, 7 days after spraying were determined using markers as standard proteins. Figure (2) show, that a new pattern of proteins, were appeared. These patterns, which have a calculated MW about 15.849 and 10 Kda, were found in SA, PS, MS, and CZ There is a difference between control and many treatments in the density of the bands, as in the pattern located between 98 and 52 KDa marker bands, the high density was found in SA, PS, MS, CI and CM bands.

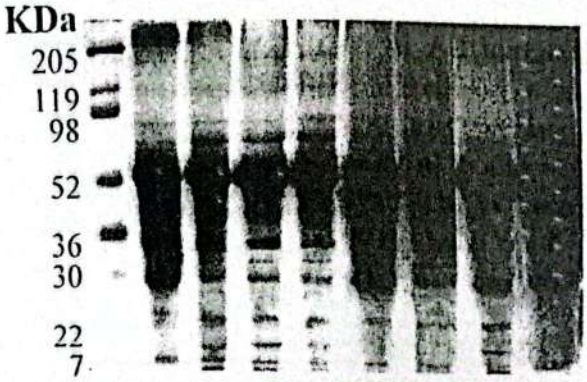


Figure (2) Electrophoretic patterns of induced cucumber plants in 12.5 % SDS-PAGE

3-Enzyme activity

3.1-Peroxidase activity:

The application with external SA (0.5%) or PS (3%) stimulate plants to produce high level of peroxidase enzyme were the absorbance was 0.511, 0.407 /10 min/g fw, which represent more than 8 and 6 fold, respectively. CI (0.5%), CZ (1%) and MS (3%) gave closed values whereas, the lowest values were for AS (0.5%) and CM (1%) (Table 3)

3.2- Chitinase activity:

SA (0.5%) or PS (3%) also induced high chitinase enzyme production in cucumber leaves where, the absorbance were 0.49 and 0.30 /1hr /1g fw, which represent more then9 and 5 fold increase than control, respectively, followed by CI (0.5%) with which, the

absorbance was high more than expected in comparison with the endogenous SA value, where gave 0.23 Ihr /1g fw. MS (0.5%) CZ (1.0%) and AS (0.5%) gave low value but more than control. CM (1%) was the only, which gave less than control (0.087/1hr/1g fw). (Table 3).

Table (3) Relationship between biochemical changes in induced cucumber plants and Virus concentration

Chemical agents	Virus conc.•	Indigenous SA (µg/g fw)	Peroxidase activity••	Citinase activity••
C *	49*	2.09**	0.060**	0.152**
SA (0.5)	0	907.2	0.511	0.49
PS (3%)	0	252	0.407	0.30
MS (3%)	18	115.2	0.222	0.208
AS (0.5%)	22	39.5	0.164	0.165
CI (0.5%)	13	144	0.275	0.231
CZ (1.0%)	17	144	0.255	0.177
CM (1.0 %)	13	43.2	0.122	0.087

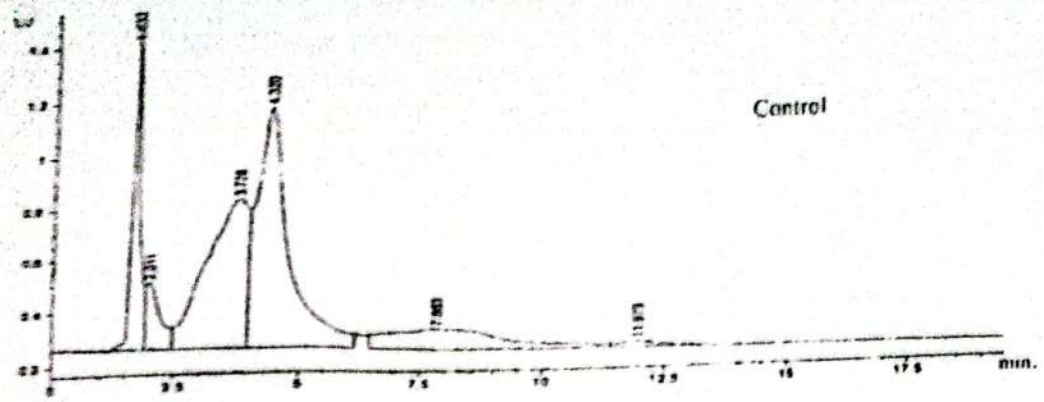
C=control, (*) Inoculated plants without spaying. (**) Sprayed plants without inoculation. (•) Virus concentration as indicated by average number of L.L. produced on C. amaranticolor. (••) Values of Peroxidase activity represent change in absorbance /1hr/g fw. while Values of Citinase activity represent change in absorbance /1hr/g fw.

Métraux & Boller (1986) showed that SAR in cucumber is correlated with increase in peroxidase and classIII chitinase activities.

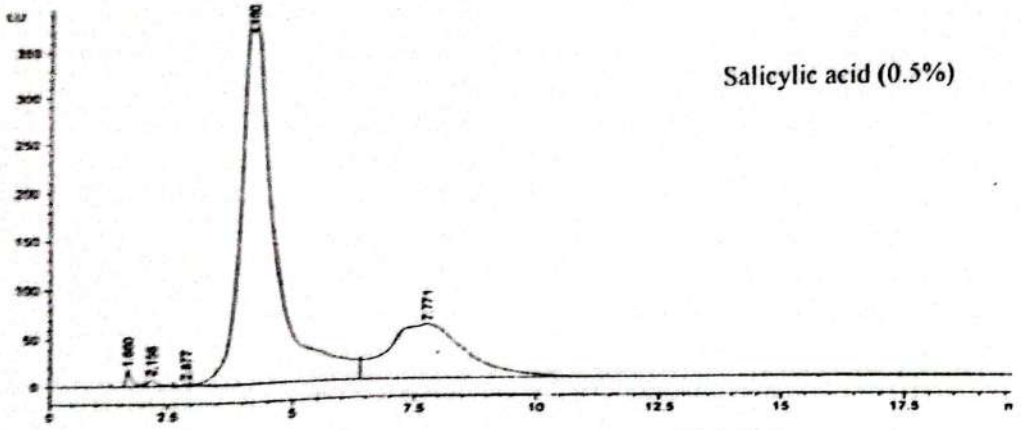
An association was found between peroxidase activity and results obtained from measurement of endogenous SA in cucumber samples. Where in cucumber, the application with exogenous SA (0.5%) and potassium sulfate (3%), which stimulated plants to produce high level of endogenous SA, produced high level of peroxidase enzyme. The results obtained for chitinase activity was in harmony with peroxidase results, where agreed with other determinations.

The results obtained from these experiments refers to the possibility of using potassium sulfate with cucumber against ZYMV because of high defense response induced, as indicated by different measurements have been taken. The difference found in endogenous SA level among different chemical materials used may due to the function of chemical elements in plants, and may each element has a relation with a group of induced gene products. So, may its need to use the chemical materials in another way, to reach high level of endogenous SA rise. In the experiment performed by Yalpani *et al.* (1991) to test the effect of temperature shifts on endogenous SA level in tobacco plants. They found that there is a graduation in endogenous SA level, when the way of treatment varied. Where less value was in control, increased gradually with exogenous SA and *Tobacco necrosis virus* (TNV) treatments, respectively, and reached the maximum value when exogenous SA and (TNV) treatments were applied together. Suggesting that the way of treatment, use inducers in groups or change in environmental factors may improve the ability of plants to produce resistance mechanisms.

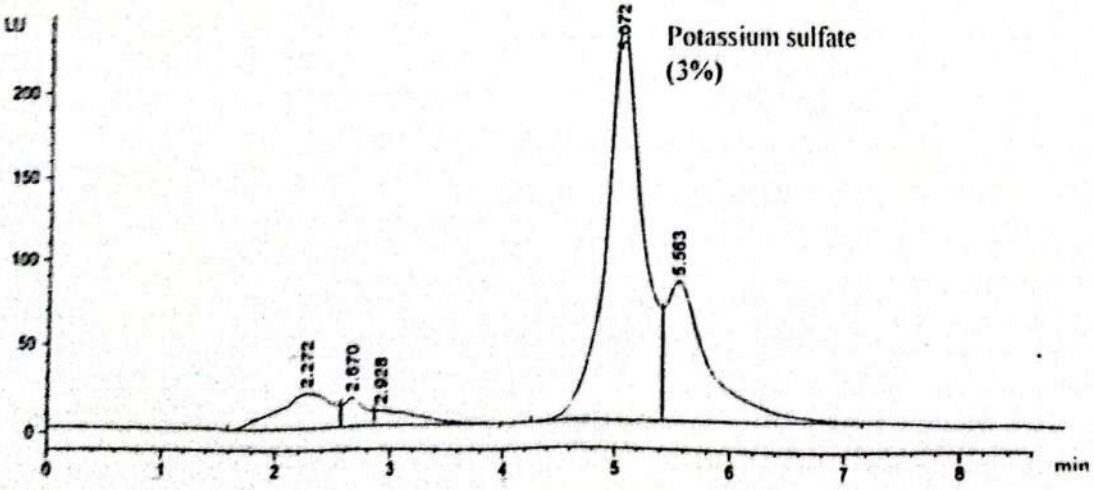
From the study of the role of potassium in plant, we find that it helps in building amino acids and subsequently proteins and activate different enzymes action. May be that explain its ability to induce SAR, which associated with production of a novel proteins and enzyme activities. Other used materials showed a medium ability to induce resistance, may be it could induce complete resistance when it used in groups or in other way.



Peak #	RetTime [min]	Width [min]	Area LU *s	Height [LU]	Area %
1	1.633	0.1433	12.42338	1.21257	11.8435
2	2.011	0.2952	5.17418	2.80269e-1	4.9327
3	3.728	0.7596	31.72729	5.69923e-1	30.2465
→ 4	4.320	0.6188	41.34717	9.20930e-1	39.4174
5	7.863	2.0403	13.00753	7.56611e-2	12.4004
6	11.979	0.7220	1.21627	2.05117e-2	1.1595

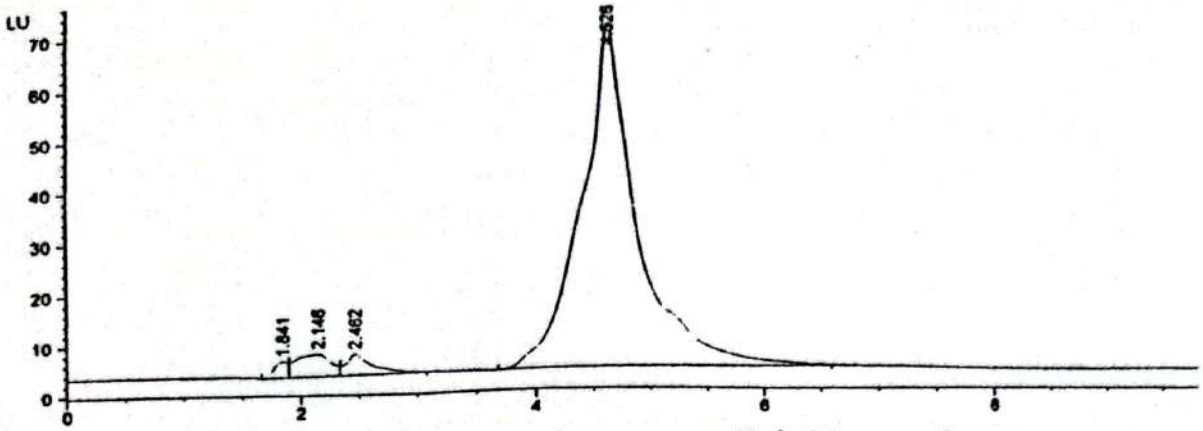


Peak #	RetTime [min]	Width [min]	Area LU *s	Height [LU]	Area %
1	1.660	0.1369	174.89815	17.69930	0.7031
2	2.156	0.2077	112.56371	7.40024	0.4525
3	2.877	0.1842	34.21473	2.59241	0.1375
→ 4	4.160	0.6627	1.80731e4	378.62469	72.6544
5	7.771	1.5433	6480.66211	56.59716	26.0524



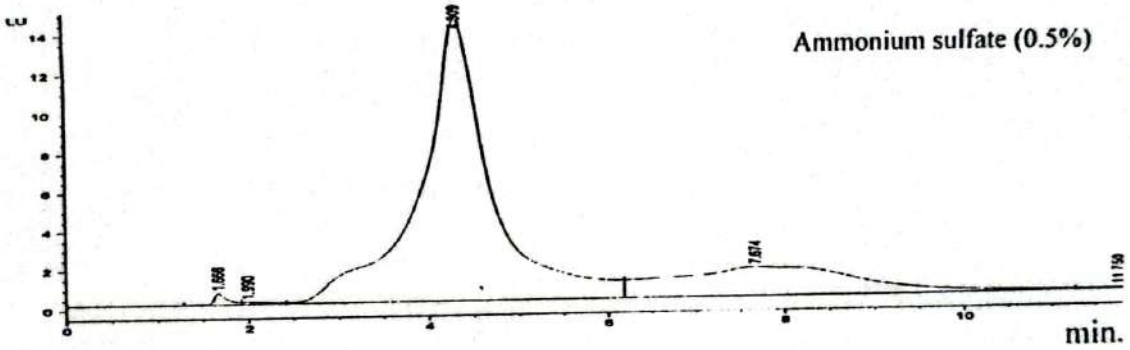
Peak #	RetTime [min]	Type	Width [min]	Area LU *s	Height [LU]	Area %
1	2.272	PV	0.4652	643.23810	19.47870	7.9693
2	2.670	VV	0.1891	216.36955	15.88946	2.6807
3	2.928	VB	0.3656	238.25919	8.23637	2.9519
→ 4	5.072	PV	0.3118	4955.44043	228.04942	61.3950
5	5.563	VB	0.3580	2018.09753	78.00245	25.0031

Magnesium sulfate (3%)



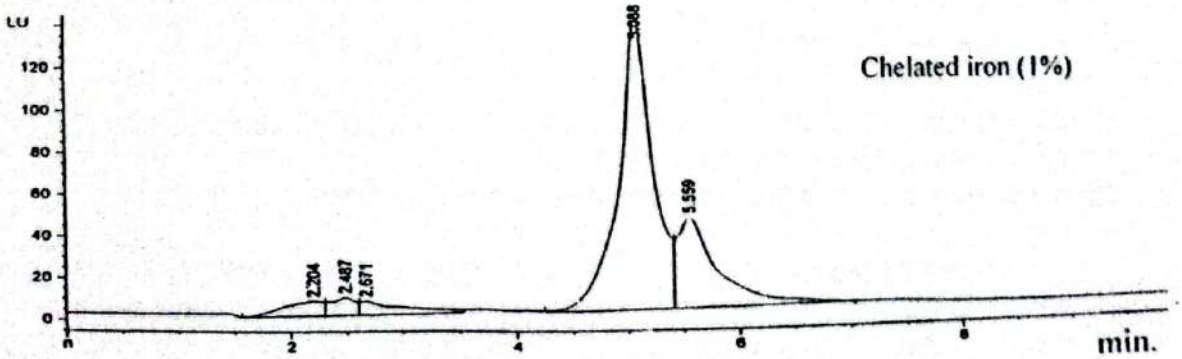
Peak	RetTime [min]	Width [min]	Area LU *s	Height [LU]	Area %
1	1.841	0.1269	26.56682	3.20525	1.0594
2	2.146	0.2723	93.01772	4.44341	3.7094
3	2.462	0.2152	68.43858	4.21736	2.7292
→ 4	4.626	0.4268	2319.61426	68.99808	92.5020

Ammonium sulfate (0.5%)

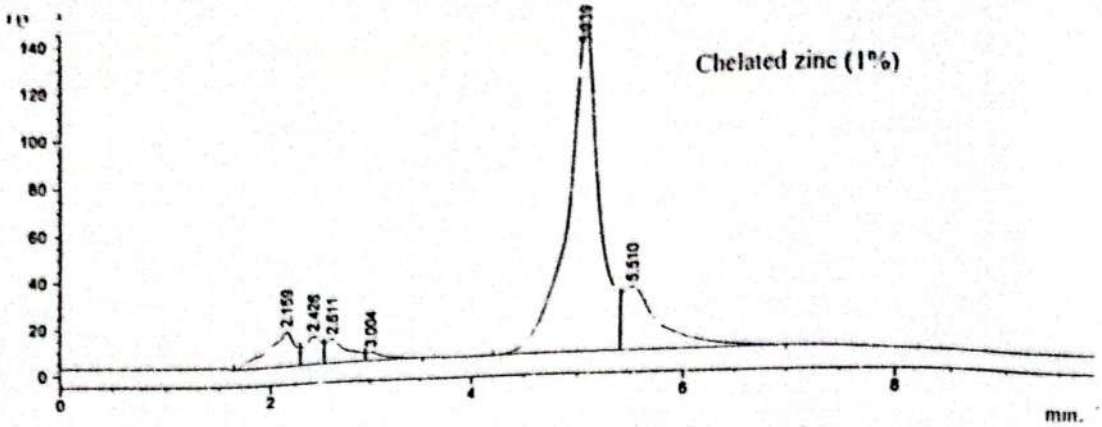


Peak	RetTime [min]	Width [min]	Area LU *s	Height [LU]	Area %
1	1.666	0.1429	5.96497	5.73622e-1	0.5920
2	1.990	0.2783	2.43375	1.14465e-1	0.2415
3	4.309	0.7330	779.43494	14.20672	77.3532
→ 4	7.674	1.9080	216.25333	1.41698	21.4616
5	11.750	0.5182	3.54416	8.74393e-2	0.3517

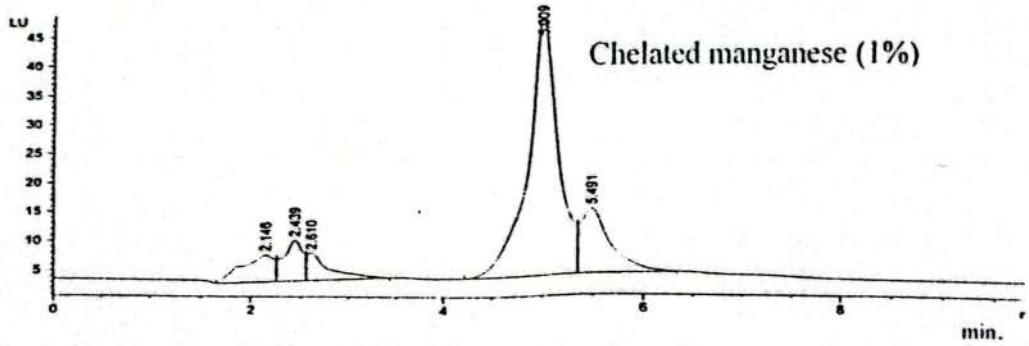
Chelated iron (1%)



Peak	RetTime [min]	Width [min]	Area LU *s	Height [LU]	Area %
1	2.204	0.3258	176.27881	6.90685	3.9672
2	2.487	0.2049	124.83560	8.23451	2.8095
3	2.671	0.3594	180.87579	6.41128	4.0707
→ 4	5.088	0.3021	2897.48096	133.93408	65.2090
5	5.559	0.3470	1063.90393	41.74524	23.9436



Peak	RetTime [min]	Width [min]	Area LU *s	Height [LU]	Area %
1	2.159	0.2401	282.91293	15.10591	6.7008
2	2.426	0.1628	143.43134	12.41928	3.3972
3	2.611	0.2092	165.94302	10.61[illegible]	[illegible]304
4	3.004	0.1817	50.54882	3.78689	1.1973
→5	5.039	0.2843	2899.60791	142.87877	68.6778
6	5.510	0.3432	679.60229	27.01046	16.0965



Peak	RetTime [min]	Width [min]	Area LU *s	Height [LU]	Area %
1	2.146	0.2835	100.24316	4.54090	7.2015
2	2.439	0.1861	92.68532	6.93798	6.6585
3	2.610	0.2254	78.41257	4.63648	5.6332
→4	5.009	0.2954	898.77051	42.67137	64.5677
5	5.491	0.2846	221.86909	10.81877	15.9391

Figure (1) HPLC quantification of endogenous SA in induced cucumber plants.
*Retention time (Ret Time) of standard SA ~ 5±1 min.
→Refer to the data of peak ranged in retention time range

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