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Studies on Salicylic Acid as a Possible Biochemical Marker for Screening Tobacco Mosaic Virus Resistant Progenies in Tobacco Breeding

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ABSTRACT: Salicylic acid (SA) has gained importance from the discoveries as an endogenous regulator of flowering in thermogenic plants. Induction of resistance to Tobacco Mosaic Virus in tobacco by exogenous application of SA and demonstration of its accumulation at the sight of necrosis following infection in tobacco plants have given a new dimension to its role in resistance. In the present study, a comparative estimation of SA in TMV resistant, tolerant and susceptible varieties was made to explore the feasibility of its use as a marker to distinguish differential response of genotypes to TMV infection. There was no difference in the basal level of SA in leaves, among varieties. However, only resistant but neither susceptible nor tolerant variety showed significant accumulation of SA after TMV inoculation and increase was 117% above the levels found in leaves of healthy plants i.e., from 0.43 to 0.94 µg/g fresh weight. To validate the practical utility of this biochemical marker in breeding programme, SA levels in TMV inoculated leaves were estimated in progenies derived from the cross involving suceptible and resistant genotypes. Higher level of SA was detected only in resistant progenies. Plants with high and low SA levels followed the typical single dominant gene controlled Mendelian ratio, comparable to the estimated visual necrotic symptoms. This proves the usefulness of salicylic acid as a supportive biochemical marker to effect selections in breeding programme.

Key words: Salicylic Acid, Marker, Tobacco Mosaic Virus, Resistance, Tolerance, Tobacco Breeding

INTRODUCTION

Salicylic acid or O-hydroxyl benzoic acid belongs to the group of plant phenolics that possess an aromatic ring bearing a hydroxyl group or its functional derivative. Plant phenolic compounds (earlier classified as secondary metabolites of minor importance) slowly gained importance and were implicated to play an essential role in regulation of plant growth, development, and interaction with other organisms (Harborne, 1980). Experimental evidences showed that, phenolic compounds function as signals in plant-microbe interactions as well, in addition to their role as chemical defenses of plants against microbes, insects and herbivores (Metraux and Raskin, 1992). Salicylic acid has been implicated as one of the key components in the signal transduction pathway, leading to plant resistance to various pathogens (Ryals *et al*., 1996; Wobbe and Klessig, 1996). Since then several reviews have been published regarding systemic acquired resistance and plant signal transduction (Raskin, 1992; Yalpani and Raskin, 1993; Ryals *et al*., 1994; Bowler and Chua, 1994). Experimentation on tobacco cultivars having varied reaction to Tobacco Mosaic Virus (TMV), Xanthi (TMV susceptible) and Xanthi-nc (TMV resistant) showed that salicylic acid concentration begins to increase during 24 to 36 hours after inoculation of TMV(Malamy *et al*., 1990). While, infection of susceptible plants fails to trigger the signal transduction pathway that leads to salicylic acid production.Findings of Yalpani *et al*. (1991) also supported the view that salicylic acid accumulation in tobacco leaf tissue and stele tissue is an integral part of the hypersensitive reaction and is sufficient for the induction of pathogenesis related proteins, commonly observed during systemic acquired resistance. Role of salicylic acid in plant immunity has been comprehensively reviewed by Baratali and Farshad (2015). These biochemical parameters in addition to their use as markers may also help in understanding therole of genetic and metabolic factors in resistance mechanism.

Biochemical markers for resistance possibly will eliminate time consuming, extensive field trials and may expedite the breeding programme. These metabolic as well as genetic markers may provide an advanced approach to plant breeding and lead the breeding programmes in most advantageous direction. In view of the above, the current study was aimed at understanding the differencial behaviour inelicitation of salicylic acid production in TMV resistant cultivar from that of susceptible and tolerant genotypes.

MATERIALS AND METHODS

A. Collection of seeds of different genotypes used in the present study

Seeds of *Nicotiana tabacum* cultivars Va 770 having resistance to tobacco mosaic virus (TMV) disease, FCH 6248 having tolerance to TMV and FCV Special, susceptible to TMV were obtained from CTRI Research Station, Hunsur. Viable seed stocks were regenerated from the cultivars grown in pots.

B. Plant culture and growth conditions and TMV inoculation

Seeds were sown in polyethylene cups of 6 cm size filled with sterilized sand / acid washed sand. These cups were kept in shade house under ambient conditions viz. 50 to 75% relative humidity, daytime temperature of 26 to 28^{o}C and night temperature of 16 to 20^{o}C and natural photoperiods. The cups were irrigated with sterile water till germination (up to 7 days), followed by half-strength Hoagland's solution (Hoagland and Arnon, 1950) for three weeks and then changed to full strength nutrient solution. The aqueous suspension of common strain of tobacco mosaic virus (TMV) isolated was inoculated on the leaves abraded with wet carborundum.

C. Salicylic acid extraction, purification and estimation

The method of salicylic acid extraction from plant tissue followed by Raskin *et al*., (1989) was adopted in the present study. One to two grams of leaf tissue was homogenated using 90% methanol at 1:25 ratio. The homogenate was centrifuged at 12000 × g for 15 min in Sorvall RC 5B refrigerated super speed centrifuge. The pellet was resuspended in 100% methanol and the homogenate was re-centrifuged at 12000 x g for 15 min. The two supernatants were combined and dried by flushing N_2 gas at 40^{o}C. The residue was resuspended in 5 ml of 1:1 (vol/vol) mixture of ethyl acetate and cyclopentane containing 1% (vol/vol) isopropanol. The top organic phase was separated and was concentrated by flushing N_2 gas.The thin layer chromatography method of salicylic acid separation as described by Rasmussen *et al*., (1991) was followed.The concentrated extract was spotted into silica gel chromatography plates of 500 µ thickness prepared using CAMAG TLC plater. The chromatograms were developed in a mixture of solvents toluene: dioxane: acetic acid at 90: 25: 5 ratio (vol/vol). The run time varied from 1 ½ to 2 hours. Salicylic acid was visualized on the plate by viewing under UV light (352 nm). The fluorescent band corresponding to salicylic acid standard was eluted from the silica gel with 1 ml of methanol and centrifuged for 5 min at 1000 rpm. The pellet was resuspended in 1 mL of methanol twice by repeating the process. The three eluates were combined and made up to final volume 3 mL with methanol and used for salicylic acid estimation.

The salicylic acid in methanol extract was quantified using spectrofluorophotometer (Shimadsu RF 5000), the procedure described by Rasmussen *et al*., (1991) with slight modification. Relative intensity was measured at excitation wavelength of λ 300 and extinction wavelength of λ 400 against methanol blank. Concentration of salicylic acid was calculated using the standard graph prepared with authentic salicylic acid standard. The quantity of salicylic acid was indicated as µg/g^{-}fresh weight.

Recovery of extractable quantity of SA from leaf tissue through the extraction & purification processwas estimated and the correction factor was used in computation.

RESULTS AND DISCUSSION

Salicylic acid (SA) was initially known only for its therapeutic property on human ailments. Its role in plant as an endogenous regulator of flowering has gained importance with the identification of its presence in thermogenic plants (Raskin *et al*., 1987). White, (1979) observed that exogenously applied SA (aspirin) induced resistance to TMV in tobacco. Demonstration of SA accumulation at the sight of necrosis following infection in tobacco plants by Malamy *et al*., (1990) & Metraux *et al*., (1990) have given a new dimension to the role of SA in resistance.

In the present study, a comparative estimation of SA in TMV resistant, tolerant and susceptible varieties were made to explore the possibility of its use as a marker to distinguish differential response of genotypes to TMV infection.

Recovery rate of SA with the method adopted for extraction, purification and detection was 39.5% in leaf, which falls with in the broad range of 34 to 62 % reported by Yalpani *et al*., (1993). Hence, mean values of triplicate assays after correction for recovery during extraction were used for estimation.

A. Effect of host - virus interaction on salicylic acid level in leaf

Salicylic acid estimation in leaves indicated that there was no significant difference in the basal level among varieties.In susceptible (FCV Special) and resistant (Va 770) varieties salicylic acid (SA) concentration was 0.37 and 0.43 µg / g fresh weight respectively in uninoculated leaves (Table 1). The data indicates that the level of SA in FCH 6248 (tolerant variety) was lower (20%) as compared to that in resistant variety. On mock inoculation there occurred no significant change in SA content irrespective of the variety. However, on TMV inoculation the salicylic acid level in leaves varied among varieties. In resistant variety, 117% higher level of SA over that of uninoculated leaf was recordedon 5th day after inoculation. This increase in resitant variety is in agreement with the results of Yalpani *et al*., (1991). These authors have shown that SA formation / accumulation results in the induction of PR proteins, which in turn ward off the infection. In their experiment, even 0.054 µg / g tissue of the plant was sufficient enough for the induction of PR proteins. We found in the present study 0.94 µg SA, which is about 17 times higher than the level required for PR protein induction. Hence, this must have enabled production of abundant PR proteins leading to resistance. Our studies again reveal that SA has a role in establishing resistance to TMV infection in tobacco.

Table 1: Salicylic acid levels in leaves of different *Nicotiana tabacum*cultivars on TMV inoculation.

Variety	**Salicylic acid ****		
	Un inoculated control	**Mock inoculated control**	**TMV inoculated**
FCV Special (S)	0.3721 ± 0.03	0.3550 ± 0.05	0.4091 ± 0.03
FCH 6248 (T)	0.3421 ± 0.02	0.2823 ± 0.04	0.3920 ± 0.07
Va.770 (R)	0.4327 ± 0.05	0.3810 ± 0.06	0.9401 ± 0.15
CD at P= 0.05	0.11		

(R) – Resistant; (T) – Tolerant; (S) - Susceptible

** µg g^{-1} fresh weight

With strong evidence from noninvasive, *in vivo* labeling of SA with $^{18}O_2$ supported by ^{14}C-labeled studies in tobacco mosaic virus resistant Xanthi-nc tobacco, Shulaev *et al*., (1995) proposed that salicylic acid is the endogenous signal in the development of systemic acquired resistance through induction of PR proteins. The hypothesis of a novel salycylhydroxamic acid sensitive mechanism (SHAM) a sensitive signal transduction pathway, which is distinct from that leading to resistance to bacteria and fungi, proposed by Chisava *et al*., (1997) further supports SA specific induced resistance to TMV in tobacco. Zhu *et al*., (2014)demonstrated that jasmonic acid and salicylic acid are required for systemic resistance response against TMV in *N. benthamiana*from virus induced gene silencing based genetics approach studies.These evidences make salicylic acid a strong candidate as a useful biochemical marker to identify TMV resistant varieties in tobacco.

The increase in susceptible and tolerant varieties on challenging with TMV was only 10% and 15% respectively and hence not very significant. TMV inoculation therefore triggered over production of salicylic acid only in the resistant variety thereby suggesting a positive role for SA in the resistance mechanism.

B. Salicylic acid as a biochemical marker in breeding experiments

The results described above implied that SA content in leaf could be used to forecast the disease resistance in breeding experiments. A higher level of SA in response to viral infection indicates resistance. In order to validate this hypothesis, breeding experiments were performed. SA levels in TMV inoculated leaves of first & second filial and back cross progenies derived from the cross FCV Special x Va 770 were estimated 3days post inoculation. The design of the experiment is given in Fig 1.

Theoretically when the susceptible variety is crossed with resistant variety the F_1,F_2 and BC progenies should follow Mendalian law of inheritance, because resistant gene is dominant over the susceptible. Accordingly, higher level of SA is expected in all the F_1 progenies as well as in the progenies derived from backcrossing with resistant parent. However progenies resulting from F_1 backcrossed with susceptible parent should have higher level of SA in only 50% of the population. The results of SA content are given in Table 2. The values were found as per prediction in all the progenies. When the F_1 was selfed, 75% of the population had increased levels of SA.

This shows that the SA content in the progeny also followed the same segregation pattern of typical Mendelian ratio of 3:1. The ratio of resistant :susceptible segregates obtained both by SA estimation on day 3 and by identifying plants based on physical appearance of necrotic local lesions on day 5 after TMV inoculation were comparable (Table 3).

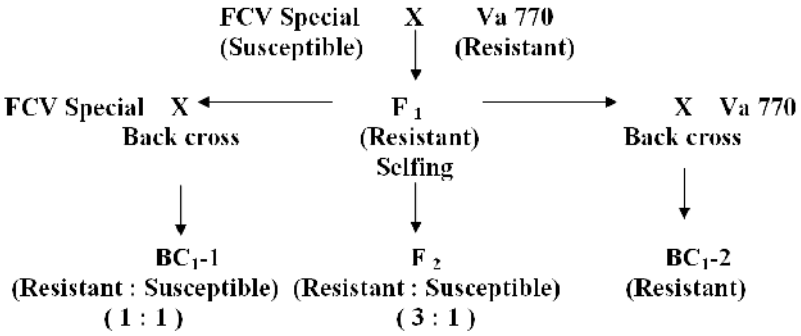

Fig. 1. Design of Breeding Experiment.

Table 2: Salicylic acid levels in viral infected leaves of different progenies of cross in breeding experiment.

Generation	Description	Disease Reaction	SA(µg /g fresh wt.)**
FCV Special	Parent – 1 (P1)	Susceptible	0.3582 ± 0.02
Va 770	Parent – 2 (P2)	Resistant	0.9360 ± 0.02
F_1	P1 crossed with P2	Resistant	0.8618 ± 0.05
F_2	F_1 selfed	Susceptible	0.3649 ± 0.04
F_2	F_1 selfed	Resistant	0.8980 ± 0.01
BC_1-1	F_1 backcrossed with P1	Susceptible	0.3652 ± 0.02
BC_1-1	F_1 backcrossed with P1	Resistant	0.8877 ± 0.01
BC_1-2	F_1 backcrossed with P2	Resistant	0.8634 ± 0.05
CD at P=0.05			0.0385

**SA content at 3 days after TMV inoculation

Table 3: Mode of inheritance in F_1, F_2 and BC generations in cross derivatives of FCV Special with Va 770 for tobacco mosaic virusdisease resistance.

Parents and Crosses	Code**	Resistance frequency		Susceptiblity frequency	
		Observed	Estimated	Observed	Estimated
FCV Special	**S**	**0**	**0**	**40**	**40**
Va 770	**R**	**40**	**40**	**0**	**0**
F1	**S x R**	**40**	**40**	**0**	**0**
F2	**S x R**	**31**	**30**	**09**	**10**
BC-1	**(S x R)S**	**19**	**20**	**21**	**20**
BC-2	(S x R)R	40	40	0	0

** S= Susceptible, R= Resistant.

In addition, the results also indicate that the estimation of SA can also be used to forecast TMV disease resistance in breeding experiments. Since plants with high and low SA levels followed the same segregation ratio as that estimated on visual necrotic symptoms,this would be a useful marker as a supportive biochemical evidence to make selections in breeding programme.

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REFERENCE

Baratali, F. and Farshad F., (2015). Salicylic acid and plant immunity. *Biological Forum – An International Journal*, **7**(1) : 79-84.

Bowler, C. and Chua, N.H., (1994). Emerging themes of plant signal transduction. *The Plant Cell*,**6** : 1529-1541.

Chisava, S., Murphy, A.M., Naylor, M. and Carr, J.P., (1997). Salicylic acid interferes with tobacco mosaic virus replication via a novel salicylhydroxamic acid sensitive mechanism. *The Plant Cell*,**9**: 547-557.

Harborne, J.B., (1980). Plant phenolics. (Ed. Bell,E.A. and Charlwood, B.V.) Encyclopedia of plant physiology new series vol.**8**: Secondary Plant Products, Springer-Verlag, Berlin, p. 329-395.

Hoagland, D.R. and Arnon, D.I.,(1950). The water-culture method for growing plants without soil.*Calif. Agric. Exp. Circ.*, 347.

Malamy,J., Carr, J.P., Klessig, D.F. and Raskin, I., (1990). Salicylic acid : A likely endogenous signal in resistance response of tobacco to viral infection. *Science,***250** : 1002-1004.

Metraux, J.P. and Raskin, I., (1992). Role of phenolics in plant disease resistance (Ed. Chet, I.) Application of Biotechnology in Plant Pathology, John Wiley and Sons, New York. p. 1-28.

Metraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B., (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science,***250**: 1004-1006.

Raskin, I., (1992). Salicylate, a new plant hormone. *Plant Physiol.*,**99** : 799-803.

Raskin, I.,Ehmann, A., Melander, W.R. and Meeuse, B.J.D., (1987). Salicylic acid- a natural inducer of heat production in Arum lilies. *Science,***237**: 1545-1556.

Raskin, I., Turner, I.M. and Melander, W.R.,(1989). Regulation of heat production in the inflorescences of anArumlily by endogenous salicylic acid. *Proc. Natl. Acad. Sci. USA*, **86** : 2214-2218.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N., (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with Pseudomonas syringae pv syringae. *Plant Physiol.*, **97**: 1342-1347.

Ryals, J., Uknes, S. and Ward, E., (1994). Systemic acquired resistance. *Plant Physiol.*, **104**: 1109-1112.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y. and Hunt, M.D., (1996). Systemic acquired resistance. *Plant Cell*, **8** : 1809-1819.

Shulaev, V., Leon, J. and Raskin, I., (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? *The Plant Cell*,**7**: 1691-1701.

White, R.F., (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology*, **99**: 410-412.

Wobbe, K.K. and Klessig, D.F., (1996). Salicylic acid – an important signal in plants (Ed. Verma, D.P.S.) Plant Gene Research Series, Signal Transduction in Plant Growth and Development, Springer-Verlag, Vienna, p. 167-196.

Yalpani, N. and Raskin, I., (1993). Salicylic acid: a systemic signal in induced plant disease resistance. *Trends in Microbiol.*,**1** : 88-92.

Yalpani, N., Shulaev, V. and Raskin, I., (1993). Endogenous salicylic acid levels correlate with accumulation of pathogenesis-related proteins and resistance in tobacco. *Phytopathology*, **83**: 702-708.

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A. and Raskin, I., (1991). Salicylic acid is a systemic signal and an inducer of pathogenesis- related proteins in virus-infected tobacco. *The Plant Cell*,**3**: 809-818.

Zhu, F., De-HuiXie, Shu Yuan, Fei Xu, Da-Wei Zhang, Hong-Hui Lin, (2014). Salicylic acid and jasmonic acid are essential for systemic resistance against tobacco mosaic virus in *Nicotiana benthamiana*. *Mol. Plant-Microbe Interact.*, **27**: 567-577.