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Modification of Antioxidant Status of Host Cell in Response to *Bougainvillea* Antiviral Proteins†

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***Bougainvillea* x*buttiana* antiviral proteins (AVPs) exhibited high antioxidant activity as measured by ferric reducing / antioxidant (FRAP) power assay. These AVPs were also found to modify activities of antioxidant enzymes like superoxide dismutase, peroxidase and catalase. The activities of superoxide dismutase and peroxidase increased, while the activity of catalase decreased in *Tobacco mosaic virus* (TMV) infected tobacco leaves. The trend was reversed when the leaves were treated with AVP alone. However, in TMV + AVP treated leaves, the activities of all the three enzymes were found to be midway between the activities obtained with other two treatments. It is therefore, suggested that *Bougainvillea* AVPs might be controlling viral diseases by scavenging reactive oxygen species as well as by altering host plant cell metabolism to maintain its antioxidant status.**

***Key words*:** *Bougainvillea* x*buttiana*, antiviral proteins, antioxidant activity, oxidoreductases.

Antiviral proteins (AVPs) are virus inhibitory substances from higher plants which are now well recognised as basic proteins. Many monocots and dicots belonging to family *Amaranthaceae, Chenopodiaceae, Euphorbiaceae, Compositae, Graminae, Solanaceae, Nyctaginaceae,* etc. are known to have such antiviral proteins. These are low molecular weight proteins (M_r 20-32 kD), with high isoelectric points and are usually stable, being resistant to denaturing agents and proteases (1).

Although, the AVPs have been well characterised and genes for a couple of such proteins have been isolated, still gaps exist in the understanding of mechanism of their action. It has been suggested that these may act either directly on virus particle, or on virus infection process or by altering host cell metabolism (2). The latter fact draws attention as plant-pathogen interactions can trigger the active production of reactive oxygen species (ROS). No doubt, the enhanced production of these species can pose a threat to the cells but these can also act as a signals for the activation of biotic stress response and defense pathways. Thus, these species out of which, H_2O_2 and $O_2^{\cdot-}$, are of special interest can be viewed as cellular indicators of biotic stress and as secondary messengers involved in the stress-response signal transduction pathway. Hence, the plant cell requires at least two different mechanisms to regulate their intercellular ROS concentrations by scavenging these species (3). This pathogen-induced oxidative burst is known to participate in the hypersensitive reaction where plant cells in the direct vicinity of an infection undergo programmed cell death in order to eliminate the most immediate source of energy and nutrients for invading pathogens (4). Major ROS scavenging mechanisms of plant include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), etc. and the balance between their activities in the cells is crucial for determining the steady state level of superoxide radicals and hydrogen peroxide (5). Besides, the local resistance mediated in terms of hypersensitive response (HR), another resistance phenomenon involved in the activation of resistance mechanism in uninfected parts of the plant is systemic acquired resistance for which salicylic acid (SA) has been suggested to act as a signal (6). Our study is an attempt to assess how *Bougainvillea* AVPs alter the host cell metabolism especially with regard to its antioxidant status, in virus infected plants.

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Abbreviations: AVPs, antiviral proteins; CAT, catalase; FRAP, ferric reducing /antioxidant power; HR, hypersensitive response; POD, peroxidase; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase; SRV, sunnhemp rosette virus; TMV, tobacco mosaic virus.

Materials and Methods

Source plant — The antiviral proteins were purified from the leaves of *Bougainvillea* x*buttiana cv* Mahara.

Virus-host combinations used — Two virus host combinations depending upon the growing season, were used to assay virus inhibitory activity of AVPs as well as for studying host-mediated response. The two tobamoviruses used were *Tobacco mosaic virus* (TMV) on *Nicotiana glutinosa* (tobacco) and *Sunnhemp rosette virus* (SRV) on *Cyamopsis tetragonoloba* (guar); both being respective local lesion hosts. Both the test plants were raised under ideal conditions in National Phytotron Facility, available at our institute. The cultures of two tobamoviruses, i.e. TMV and SRV were maintained on their respective systemic hosts, tobacco (*Nicotiana tabacum* var NP 31 or Samsun NN) and sunnhemp (*Crotolaria juncea*) in insect-free glass house.

Preparation of virus inoculum — Virus inoculum was prepared as described by Narwal *et al* (7). The virus inhibitory activity was calculated in terms of per cent inhibition of lesion formation using following formula :

Per cent inhibition = [(C - T) / C] x 100

where, C = Average number of lesions on control plants
T = Average number of lesions on treated plants

Purification of antiviral proteins from Bougainvillea leaves — Antiviral proteins present in the leaf extract of *Bougainvillea* x*buttiana* were purified by following protocol of Narwal *et al* (7).

Estimation of antioxidant activity — The antioxidant activity of purified antiviral proteins was estimated by ferric reducing / antioxidant power (FRAP) assay method as given by Benzie and Strain (8). 100 µl of antiviral protein samples were mixed with freshly prepared 3.4 ml of FRAP reagent. The reaction tubes were kept at room temperatre (25 °C) for different time periods starting from 2 to 8 min. After each incubation period, absorbance of the reaction mixture was measured at 593 nm. FRAP reagent alone was used as blank. Standard curve was prepared by taking different concentrations of freshly prepared ammonium ferrous sulphate in the range of 100-1000 µmol l^{-1}. FRAP value obtained for antiviral proteins was expressed as µmol equivalent of ferrous ions (Ferric reducing power).

Nicotiana glutinosa plants were treated with buffer (as control), TMV alone, *Bougainvillea* AVP alone, and AVP + TMV. For each treatment 2-3 plants per pot were taken. The leaf samples were collected at 0, 1, 2, 3, 4 and 5 days after treatment, and frozen in liquid nitrogen. Activities of antioxidant enzymes like superoxide dismutase , catalase and peroxidase were assayed in the extracts of frozen leaf samples prepared differently, as detailed below.

Superoxide dismutase — Superoxide dismutase was assayed according to modified method of Beauchamp and Fridovich (9). The activity of SOD was estimated on the basis of its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT). The absorbance was read at 560 nm and the 50% inhibition was taken equivalent to one unit of SOD. The activity of SOD was expressed as units g^{-1} fr wt leaf min^{-1}.

Peroxidase — The assay of peroxidase was based on the rate of formation of guaiacol dehydrogenation product. The activity in the leaf extract was assayed by the method of Zeislin and Zaken (10). The change in absorbance was recorded at 470 nm immediately after adding H_2O_2 over a period of 3 min with an interval of 30 sec. The activity of peroxidase was expressed as units g^{-1} fr wt leaf min^{-1} by taking a change in absorbance of 0.1 as 1 unit of enzyme.

Catalase — The catalase activity was assayed according to Teranishi *et al* (11) with minor modifications. The absorbance was recorded at 410 nm and a blank was prepared using heat killed enzyme in assay mixture. Catalase activity was expressed as µmol H_2O_2 g^{-1} fr wt leaf min^{-1}.

HPLC analysis of endogenous SA in AVP treated plants — To find out the possibility of involvement of salicylic acid in viral infection inhibition by AVPs, guar plants at 3-4 leaf stage were treated with *Bougainvillea* AVP alone, AVP + SRV and SRV alone. Buffer treated plants were taken as control. For each treatment, 2-3 plants per pot were taken in three replicates. The leaves were collected at 15 h and 30 h time interval, after the treatment. The procedure used for SA extraction was as described by Yalpani *et al* (12). HPLC analysis was performed on Thermo Separation Product model Spectra System P2000 equipped with, a variable wavelength UV-VIS detector, a Rheodyne injector (20 µl loop) and connected to a Datajet reporting integrator. Separation of SA was detected at 280 nm on a Lichosorb C_{18} stainless steel column (250 mm x 4.6 mm i.d.) with a mobile phase flow rate of 0.8 ml min^{-1}. SA was separated isocratically with 23 % methanol (v/v) in 20 µM sodium acetate buffer (pH 5.0). The results were expressed as µg SA g^{-1} fr wt leaf.

Statistical analysis — The data obtained from enzyme assays and HPLC analysis of salicylic acid were analysed statistically using factorial completely randomized design (CRD). The values obtained were means of three replicates.

Results

The present study was, undertaken with a view to understand the mechanism of action of *Bougainvillea* antiviral proteins in terms of their effect on host cell metabolism. The antiviral proteins were purified to 36.8 fold from the leaves of *Bougainvillea* x*buttiana.* The purified preparation when analysed on 12 % SDS-PAGE gave two closely spaced bands corresponding to two polypeptides of M_r 33 and 28 kD (results not shown).

Antioxidant activity — The antiviral proteins when applied along with virus or before virus inoculation, inhibited the viral infection and local lesion formation (results not shown). As viral infection is accompanied by oxidative burst, these AVPs may be controlling this oxidative burst by quenching the free radicals owing to their antioxidant activity. Hence, the *Bougainvillea* x*buttiana* AVPs were tested for their ferric reducing/antioxidant power (FRAP) value. The aqueous solution of AVPs (100 µmol / 100 µl) was used for measuring FRAP value with reaction time as 4 min. The results presented in Table 1 showed that for AVPs, the FRAP value was found to be 461 as against a value of 682 for equimolar ascorbic acid, a standard antioxidant. This indicates that the FRAP value of *Bougainvillea* AVPs is nearly 68% of ascorbic acid.

Table 1. Antioxidant activity of *Bougainvillea* antiviral proteins

Sample*	FRAP value [µmol equivalent of Fe^{II}]
Ascorbic acid	682 ± 10.6
Antiviral protein	461 ± 16.5

*The concentration of ascorbic acid as well as AVP was ~100 µmol/100 µl.

Variation in the activities of antioxidant enzymes — Viral infection on a local lesion host results in hypersensitive response, and this response is associated with changes in the activities of various oxidoreductases present in the host cell. In the present investigation, the activities of three enzymes namely SOD, POD and CAT in TMV infected tobacco leaves as affected by AVP treatment were studied, and expressed on per gram fresh weight basis. When the tobacco leaves were treated with TMV alone, the overall activity of SOD increased significantly as compared to control (buffer treated). On the contrary, when the leaves were treated with AVP alone there was a significant decrease in the activity of the enzyme (Table 2). However, when the leaves were treated with TMV + AVP, a significant decrease in activity was observed as compared to plants treated with TMV alone, and the value was mid-way between the values obtained for TMV alone and AVP alone treated plants when the overall effect of different treatments is compared.

Table 2. Activity of superoxide dismutase in TMV infected tobacco leaves as affected by AVP treatment

Days	Activity (Units g^{-1} fr wt min^{-1}) x 10^{-3}			
	Treatment			
	Control	TMV alone	AVP alone	AVP + TMV
0	16.33	19.00	20.33	15.33
1	15.00	17.00	17.33	13.66
2	15.00	26.33	9.33	22.66
3	19.66	21.00	16.66	23.66
4	20.66	21.66	18.66	20.00
5	22.33	20.66	17.33	16.66
Mean treatment	18.16^{b}	20.94^{a}	16.61^{c}	18.72^{b}

Note: Mean values with different superscripts are statistically different.

The activity of peroxidase too vary significantly with different treatments (Table 3).The activity increased significantly to about 3.4 fold when tobacco leaves were

Table 3. Activity of peroxidase in TMV infected tobacco leaves as affected by AVP treatment

Days	Activity (Units g^{-1} fr wt min^{-1})			
	Treatment			
	Control	TMV alone	AVP alone	AVP + TMV
0	8.36	26.32	9.74	7.74
1	10.53	47.12	13.24	15.30
2	12.86	64.48	10.06	31.76
3	7.58	23.52	11.44	64.00
4	14.00	45.44	20.3	24.80
5	15.22	27.12	21.83	14.14
Mean treatment	11.42^{d}	39.00^{a}	14.43^{c}	26.29^{b}

Note: Mean values with different superscripts are statistically different.

treated with TMV alone. In this case, the peak activity was observed on 2nd day. When the leaves were treated with AVP alone, the activity increased to only 1.2 fold as compared to control. However, when the leaves were treated with AVP + TMV, the activity decreased significantly as compared to TMV treatment, and it was mid-way between TMV alone and AVP alone treatments.

For catalase activity however, the trend reversed as there was a significant decrease (57.5%) in catalase activity when tobacco plants were treated with TMV alone, as shown in Table 4. On treatment with AVP alone, a slight but significant increase in the activity was noticed. When the plants were treated with AVP + TMV, the activity increased to about 1.5 fold compared to the activity obtained with TMV alone treated leaves.

Table 4. Activity of catalase in TMV infected tobacco leaves as affected by AVP treatment

Days	Activity (μmol H_2O_2 g^{-1} fr wt min^{-1})			
	Treatment			
	Control	TMV alone	AVP alone	AVP + TMV
0	51.70	51.66	46.55	67.43
1	42.38	9.01	55.47	29.58
2	69.90	42.42	73.70	46.52
3	58.84	10.94	58.33	16.82
4	43.06	9.44	57.58	28.81
5	42.37	7.20	56.31	14.13
Mean treatment	51.36[b]	21.78[d]	57.99[a]	33.88[c]

Note: Mean values with different superscripts are statistically different.

A similar trend was observed when the activities of all the three enzymes were expressed on per mg protein basis (results not shown).

Salicylic acid level in SRV infected guar leaves — Induction of systemic resistance is a feature of *Bougainvillea* AVPs, and salicylic acid is known to be a signal molecule for systemic resistance. The guar plants treated with AVPs alone showed significant increase in the level of endogenous salicylic acid as compared to control (buffer treated) plants. However, in SRV alone or AVP + SRV treated plants there was no significant change in the level of salicylic acid as compared to control (Table 5).

Table 5. Level of salicylic acid at two time intervals in SRV infected guar leaves as affected by AVP treatment

Treatment	Salicylic acid (μg g^{-1} fr wt leaf)		Mean treatment
	15 h	30 h	
Control	8.33	2.68	5.05[b]
AVP alone	7.63	7.82	7.72[a]
AVP + SRV	6.14	4.04	5.09[b]
SRV alone	4.99	4.55	4.77[b]
Mean hour	6.77[a]	4.77[b]	

Note: Mean values with different superscripts are statistically different.

Discussion

Viral inhibitors of variable potency, that occur in non-host plants naturally, can be utilised for combating viral diseases. No doubt, these endogenous inhibitors have been well characterised with regard to their nature, but the understanding of their mode of action is not complete. This is still an active area of research, and before their potential for developing transgenics is explored, it is necessary that the mechanism of action of these antiviral substances is clearly understood. The present study, therefore, is an attempt towards understanding the possible mechanism by which antiviral proteins of *Bougainvillea* cause inhibition of viral infection (7, 13).

AVPs cause inhibition of viral infection by suppressing the formation of local lesions. There is a possible role for these AVPs in controlling oxidative burst. This holds true as *Bougainvillea* antiviral proteins showed strong antioxidant activity which indicated that these might have a role as scavenger of reactive oxygen species (ROS) generated during virus infection. Similar results have also been shown with *Celosia* AVP (14). Amino acids like Trp, Met, His and Lys (15) as well as glycoproteins and glycopeptides have also been shown to have antioxidant activity (16). The results obtained in present study draw further support from the fact that *Bougainvillea* antiviral proteins too are glycoproteins and are rich in basic amino acid lysine (7).

Biotic and abiotic stresses often result in production of reactive oxygen species like superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) in plants. These ROS are critical features of host defense responses which involve synthesis of antioxidants like ascorbate and glutathione (17). The role of ROS in plant-

microbe interactions has been clearly established in recent years. It has been suggested that modification of ROS levels by antioxidant enzymes such as superoxide dismutase, catalysing dismutation of $O_2^{\cdot-}$, and catalase , which converts H_2O_2 to water and oxygen may be integral to development of antimicrobial defense. Increased H_2O_2 levels may lead to the induction of systemic acquired resistance, which develops in non-infected parts of the plant (18).

In the present study, the *in vitro* activity profiles of three antioxidant enzymes were studied in the leaves of tobacco at different intervals after treating tobacco plants with TMV, AVP or TMV + AVP in combination. When AVPs are applied prior to virus inoculation or in combination with virus, there was suppression of necrotic lesion formation in local lesion host. This might have been due to change in the antioxidant status of the cell as a result of the variations in activities of enzymes SOD, CAT and POD that are responsible for warding off active oxygen species in the cells (19).

Superoxide dismutase catalysing the breakdown of superoxide anion provides the first line of defense against oxygen toxicity. The increase in the activity of SOD on TMV infection noticed in the present study indicates the increased dismutation of superoxide anion leading to formation of H_2O_2 which may function as intra or intercellular signal molecule (20). Similar increase in the activity of SOD on TMV inoculation has been reported by Legrand *et al* (21). However, there was a decrease in the activity of SOD on treatment with AVP alone, and it did not change much when the leaves were treated with both AVP and TMV taken together. The decline in the catalase activity noticed in the present study on TMV infection, is in accordance with a decrease observed in earlier studies carried out by Neuenschwander *et al* (22). Evidences indicate that ability to control H_2O_2 levels is one of the factors that contribute to the resistance against various stresses. Fine tuning of H_2O_2 levels is must for such response and this tuning is further maintained by catalase and peroxidase.

Peroxidase catalyses the oxidation of cellular components by H_2O_2 or hydroperoxides. In this study, the activity of POD also increased when tobacco plants were inoculated with TMV alone. Similar results have been reported with other plant-virus interactions like tobacco and *Tobacco necrosis virus*, bean and *Peanut mottle virus*, and in cucumber infected with *Cucumber mosaic virus* (23). All these interactions result in hypersensitive response and development of necrotic lesion, a system similar to the one selected in present study. However, the increase in activity due to TMV infection contrasts the treatment with AVP alone. Thus, the present study suggests that on TMV infection, the general decline in CAT activity and simultaneous increase in SOD activity would help in increased accumulation of H_2O_2 which in turn would support the activity of POD. Since, the effect observed on AVP treatment for all the three enzymes antagonises the treatment with TMV alone, it can be expected that AVPs regulate the level of H_2O_2 to a non-toxic concentration. This is supported by the observation that H_2O_2 is non-toxic or even beneficial at moderate concentration but toxic at high concentration (24).

Moreover, when the effect of AVP + TMV is observed, the activities of three enzymes SOD, CAT, POD lie midway between the two other treatments, i.e. TMV alone and AVP alone. Thus, the overall results obtained can be well explained by the hypothesis that *Bougainvillea* AVPs act as strong antioxidant agents thereby, these themselves may scavenge the active oxygen species or free radicals resulting from virus infection, and additionally or alternatively these AVPs may trigger plant defense mechanisms by maintaining balance of antioxidative enzymes to scavenge these species.

Salicylic acid is known to play an important role in systemic acquired resistance, which protects the plant from further attack by pathogens (25). There is a possibility that the role of AVP in inhibiting viral infection may be mediated *via* SA. The increased concentration of SA in plants treated with AVPs indicate that it may be acting as messenger carrying the signal from cell receptors where AVPs might be acting, and finally switching on various genes for pathogenesis related (PR) proteins (6). SA has been proposed as a natural inducer of PR proteins and its high endogenous level has been reported during both HR as well as SAR (26). In our earlier study *Bougainvillea* AVP has been shown to induce, actinomycin D reversible systemic resistance in guar plants against SRV (13). Since in this study, an increase in the endogenous level of SA, on application of AVP to guar leaves was significant over other treatments, it shows the possibility of SA-dependent signalling cascade that may lead to SAR against SRV.

Thus, the present study suggests that the antiviral proteins of *Bougainvillea* may maintain the antioxidant status of plant host cell to the extent that it results in

suppression of viral disease. This may be, partly due to their ROS scavenging action owing to antioxidative activity and partly due to their effect on the activities of antioxidative enzymes.

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