



Plant Archives

Journal homepage: <http://www.plantarchives.org>DOI Url : <https://doi.org/10.51470/PLANTARCHIVES.2026.v26.no.2.065>

HARNESSING SYSTEMIC ACQUIRED RESISTANCE INDUCERS TO ENHANCE DISEASE RESISTANCE AGAINST POTATO LEAF ROLL VIRUS (PLRV) IN LOCAL LANDRACE OF POTATO OF ASSAM, INDIA

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(Date of Receiving-24-05-2026; Date of Revision-30-07-2026; Date of Acceptance-20-08-2026)

ABSTRACT

Potato Leaf Roll Virus (PLRV) is a persistent and devastating viral disease that significantly impacts potato yields. In India, where the incidence of PLRV is alarmingly high, a study was conducted on local potato landraces in Assam to develop a management strategy for this disease. Given the degenerative effect of PLRV on potato tubers, the study focused on harnessing the potential of Systemic Acquired Resistance (SAR) elicitors in plants. Three SAR inducers - salicylic acid (SA), S-Methyl 1,2,3-benzothiadiazole-7-carbothioate (BTH), and 2,6-dichloroisonicotinic acid (INA) - were evaluated at three concentrations (0.5 mM, 1.5 mM, and 2.5 mM) in a field experiment. The SAR inducers were exogenously applied to potato plants at 30 days after germination, with two subsequent applications at 7-day intervals. Notably, BTH at 1.5 mM significantly reduced PLRV incidence by 87.03% compared to the control, a finding supported by biochemical analyses using DAB and PPO assays.

Key words: landrace, potato, SAR inducers

Introduction

Potatoes and their wild relatives, comprising the largest genus *Solanum* with approximately 1500-2000 species, are widely distributed globally. In North East India, particularly in Assam, a unique species of potato is cultivated, characterized by smaller size compared to commercial potatoes. These local landraces are well adapted to the sub-Himalayan foothills region and exhibit round and nut-shaped tubers. (Chowdhury and Datta, 2020) The local potato growers in Assam cultivate these landraces due to their high market demand and value. However, the availability of these potatoes is limited to certain seasons, which creates a challenge for meeting the demand throughout the year. Despite their high market value and preference among customers, these potatoes are seasonal and susceptible to Potato Leaf Roll Virus (PLRV), which compromises tuber quality. Moreover,

the small-sized potatoes are highly susceptible to PLRV, which further reduces their yield and quality.

PLRV is the type species of the genus *Potyvirus*, in the family *Luteoviridae*, is transmitted by aphids and infects approximately 20 solanaceous species, causing leaf rolling and stunting. (Talianky *et al.*, 2003). The continuous use of infected seed stocks has led to degeneration of these potatoes. To overcome these challenges, researchers have been exploring various strategies to control PLRV and improve the yield and quality of these potatoes. One such approach is the use of SAR, which has been shown to be effective in inducing broad-spectrum resistance against various pathogens. (Kuc, 2001) By leveraging SAR, potato growers can reduce the use of chemicals and develop sustainable farming practices. To control disease, strategies such as chemical-induced disease resistance have been explored,

leveraging systemic acquired resistance (SAR) to achieve broad-spectrum resistance. (Chaturvedi *et al.*, 2008; Wendehenne *et al.*, 2014) Exogenous application of salicylic acid and analogs has been shown to activate defense responses, priming cells for enhanced defense elicitation against pathogens. (Tuzun and Kuc, 1985)

Furthermore, the use of salicylic acid and its analogs has been shown to be effective in activating defense responses in potatoes. Salicylic acid is a natural plant hormone that plays a key role in regulating defense responses in plants. By applying salicylic acid or its analogs, potato growers can induce defense responses in their crops, making them more resilient to PLRV and other pathogens. (Gao *et al.*, 2015) This approach has been shown to be effective in reducing the incidence of PLRV and improving the yield and quality of potatoes. Overall, the development of sustainable farming practices that leverage SAR and salicylic acid could help improve the productivity and sustainability of potato cultivation in Assam and other regions. The objectives include- a) to study the effectiveness and optimal dose of foliar applications of SA, BTH and INA inducing SAR against PLRV; b) to identify the best SAR inducer against PLRV.

Materials and Methods

Use of SAR inducers- The experiment was carried out in the Instructional cum Research (ICR) Farm, AAU during 2020 and 2021. All the recommended practices of cultivation were followed according to the package of practices of Assam Agricultural University, Jorhat, Assam, India. The experimental plot was laid out in randomized block design (RBD) for 10 treatments with 3 replications and for that 30 numbers of plots having size of 6 square meter (3m x 2m) were prepared for planting potato. The potato tubers were sown on 11th November, 2020 and 2021. The SAR inducers used in this experiment were salicylic acid (SA) (Himedia, PCT0826), S- methyl 1, 2, 3-benzothiadiazole-7-carbothioate (BTH) (Sigma-Aldrich, CAS Number 135158-54-2) and 2, 6-dichloroisonicotinic acid (INA) (TCI, CAS-RN5398-44-7, Product Number D3345) in different concentrations. A stock solution of 1 M was prepared at first for each of the SAR inducers. The inducers were at first dissolved in the required solvents to prepare the solution. SA and BTH was dissolved in acetone, INA was dissolved in methanol. Thereafter the volume makeup was done in the 1M stock solution. The working concentration of the SAR inducers used was mentioned in Table 1. The working solution was added with Tween-20 (0.03%, v:v). In the control plots, water plus Tween-20 (0.03%, v:v) was sprayed. The spray was conducted in a phytosanitary

Table 1: Treatments used for application of SAR chemicals.

Treatment	Treatment description
T ₁	0.5mM SA
T ₂	1.5 mM SA
T ₃	2.5 mM SA
T ₄	0.5mM BTH
T ₅	1.5 mM BTH
T ₆	2.5 mM BTH
T ₇	0.5mM INA
T ₈	1.5 mM INA
T ₉	2.5 mM INA
T ₁₀	Control

backpack sprayer in all the treatments until runoff. The spray was applied to plants at 30 days after germination at 7 days interval for 3 times. The plants were observed regularly for symptoms.

The SAR inducers treated plants were subjected to molecular analysis for the presence of PLRV. For the occurrence of the disease, the source of infection was natural vector transmission of PLRV. For this purpose RNA extraction, PCR analysis and gel electrophoresis were conducted on the potato plants.

Biochemical analysis for validation of SAR assay- Two different biochemical tests were conducted on the SAR inducers treated plants to observe the activation of SAR by the release of certain chemicals in plants.

3, 3- Diaminobenzidine (DAB) assay - Potato leaves were collected from the field treated with SAR inducers after 24 hours of spraying. DAB assay was done to detect the accumulation of hydrogen peroxide by dark brown staining. For the detection of hydrogen peroxide the protocol developed by Liu *et al.*, (2012) was employed.

Procedure- DAB staining solution was freshly prepared. Sterile distilled water was prepared at a pH of 3.6 by addition of 0.1 N HCl. To this water 3, 3-diaminobenzidine was added. (10 mg per 10 ml of water) DAB was allowed to dissolve in the solution by incubating at 37 °C for 1 hour with vigorous shaking. The samples were then placed in sterile petriplates and 10 ml of DAB solution was added per sample and incubated at 37 °C overnight. The next day the excess of DAB solution was discarded and rinsed with sterile water. The samples were then kept in fixative solution (ethanol/ acetic acid 3:1, v/v) and boiled for 15 minutes to remove the chlorophyll. The samples were then removed from the fixative solution and washed with distilled water and transferred to sterile petri plates. The samples were then transferred to lactoglycerol solution (lactic acid/ glycerol/ water 1: 1: 1, v/v/v) for one hour and then finally visualized under white light background.

Table 2: Effect of SAR inducers on the incidence of PLRV.

Treat- ments	15 days post treatment			30 days post treatment		
	Symptomatology	Disease incidence (%)	Percent disease reduction over control (%)	Symptomatology	Disease incidence (%)	Percent disease reduction over control (%)
T ₁	Leaf rolling, mosaic, purple colouration on leaves	23.59 (29.06 ^{bc**})*	67.52	Leaf rolling, mosaic, purple colouration on leaves	31.48 (34.12 ^{b**})*	65.99
T ₂		19.42 (26.15 ^{cd})	73.26		21.75 (27.80 ^{cd})	76.50
T ₃		27.28 (31.49 ^b)	62.44		33.79 (35.54 ^b)	63.50
T ₄		15.70 (23.34 ^d)	78.38		19.44 (26.16 ^{de})	79.00
T ₅		8.33 (16.77 ^e)	88.53		12.00 (20.27 ^e)	87.03
T ₆		15.23 (22.97 ^{de})	79.03		16.17 (23.71 ^{de})	82.53
T ₇		26.36 (30.89 ^{bc})	63.71		29.14 (32.67 ^{bc})	68.52
T ₈		16.19 (23.72 ^d)	77.71		20.82 (27.15 ^d)	77.50
T ₉		27.31 (31.50 ^b)	62.41		29.16 (32.68 ^{bc})	68.50
T ₁₀		72.66 (58.47 ^a)	-		92.58 (74.20 ^a)	-
SD		20.14	23.96		22.87	24.70
CD (p=0.05)	-	7.17	-	-	7.84	-
*Figures in the parenthesis represent angular transformed values **Values superscripted with same letters are significant at 5 per cent T ₁ – 0.5 mM SA; T ₂ – 1.5 mM SA; T ₃ – 2.5 mM SA; T ₄ – 0.5 mM BTH; T ₅ – 1.5 mM BTH; T ₆ – 2.5 mM BTH; T ₇ – 0.5 mM INA; T ₈ – 1.5 mM INA; T ₉ – 2.5 mM INA; T ₁₀ – control						

Spectrophotometric assay of Polyphenol oxidase (PPO) activity

Extraction of Polyphenol oxidase (PPO) - Potato leaf tissues (2 g) of different treatments were taken separately in a pre-cooled mortar and pestle with 2 ml of phosphate buffer (pH 6.0) and ground. The homogenate was then centrifuged at 20,000 rpm for 15 minutes at 4 °C. The supernatant was used as the source of enzyme.

Estimation of Polyphenol oxidase (PPO) - PPO activity was measured by using the method with modifications as described by Mahadeva and Sridhar (1986). 0.5 ml of enzyme extract, 2.0 ml of phosphate buffer (pH 6.0) and 0.5 ml of substrate pyrogallol solution were taken in a quartz cuvette and mixed. The cuvette was inverted to mix thoroughly and immediately the initial reading was taken at 495 nm. Further readings were taken after every 5 minutes interval. Another cuvette filled with 3 ml of phosphate buffer was used as blank.

Molecular analysis of PLRV- To identify the different potato viruses infecting the land races of potato they were subjected to molecular analysis by extraction of RNA, polymerase chain reaction (PCR) and gel electrophoresis.

a) Plant RNA isolation- Total genomic RNA from healthy and virus infected potato plants were extracted by following CTAB (Cetyl Trimethyl Ammonium Bromide) method of Lodhi *et al.* (1994).

b) Two step RT-PCR amplification- RT-PCR combines reverse transcription of RNA into DNA *i.e.*; complementary DNA (cDNA) and amplification of specific DNA targets using PCR. The RNA template was converted to cDNA by using the Prime Script™ 1st strand cDNA synthesis kit (TaKaRa-6110A) as per manufacturer's protocol. For the processing of PCR, EmeraldAmpGT (TaKaRa RR310A) Master Mix was used. The working volume of PCR master mix included Emerald Master Mix (4.0 µl), forward primer (0.5 µl), reverse primer (0.5 µl), sterile distilled water (4.5 µl) and template cDNA (0.5 µl) making a total volume of 10.0 µl. The primer pair used for PLRV was F: ATGAGTACGGTCGTGGTTAAAGG; R: GGCCTGCAGGCTATTTGGGGTTTTG as mentioned in Awasthi *et al.*, 2014.

c) PCR Cycle- The PCR cycles were performed in a thermal cycler (Aligent SureCycler 8800, Aligent Technologies, USA) and the cycling protocol used for the detection of the viruses included 94 °C for 2 minutes denaturation. The PCR amplification was carried out for 30 cycles under the following conditions: denaturation at 94 °C for 30 seconds, annealing at 53 °C for 30 seconds, extension at 72 °C for 30 seconds and a final extension at 72 °C for 7 minutes and finally stored at 4 °C.

d) Agarose gel preparation and analysis of PCR products- The PCR products were resolved on 1.5 per cent agarose gel in Tris EDTA (TAE) containing ethidium bromide (Sambrook and Russell, 2001). The gel was observed under an UV-transilluminator Gel-doc to observe the amplified bands.

Results and Discussion

The impact of Systemic Acquired Resistance (SAR) inducers on the incidence of Potato Leaf Roll Virus (PLRV) is presented in Table 2. The percentage incidence was determined through RT-PCR analysis. The results show that the highest incidence of PLRV (72.66%) was observed in the control treatment (T_{10}) at 15 days after the application of SAR inducers. In contrast, the treatments that received SAR inducers exhibited significantly lower disease incidence, with the highest levels observed in treatments T_9 (2.5 mM INA), T_3 (2.5 mM SA), and T_7 (0.5 mM INA), at 27.31%, 27.28%, and 26.36%, respectively. Notably, the lowest incidence of PLRV (8.33%) was observed in treatment T_5 (1.5 mM BTH). Further observations at 30 days after spraying revealed that the disease incidence in the control treatment (T_{10}) increased to 92.58%. Among the treated plants, the highest disease incidence was observed in treatment T_3 (2.5 mM INA) at 33.79%, while the lowest incidence was observed in treatment T_5 (1.5 mM BTH) at 12.00%. The reduction in disease incidence over the control treatment was highest in treatment T_5 (1.5 mM BTH) at 87.03% and lowest in treatment T_3 (2.5 mM SA) at 63.50%.

The detection of PLRV in SAR chemical treated plants were done through PCR analysis and observed under gel documentation and represented in Fig. 1. It was observed in the gel documentation the presence of

PLRV in the samples as they had a band size of 629 bp in different treatments.

According to Lawton *et al.*, (1996), the chemical BTH activates a parallel signal transduction pathway to SA, leading to Systemic Acquired Resistance (SAR) and the subsequent induction of Pathogenesis-Related (PR) proteins and disease resistance. Nie (2006) demonstrated that exogenous application of salicylic acid (SA) suppresses Potato Virus Y (PVY) by increasing necrosis in leaves and nodes, with a 1 mM concentration of SA reducing PVY incidence. However, Sillero *et al.*, (2012) found no significant difference between SA and BTH in their effectiveness against disease. They observed a reduction in disease severity and Area Under the Disease Progress Curve (AUDPC) values when BTH and SA were applied in the field at concentrations between 1 mM and 5 mM, effectively controlling *Ascochyta* blight in faba bean. Notably, the effectiveness of both chemicals decreased at concentrations above 2.5 mM, consistent with our experimental findings.

Biochemical analysis for assay of SAR inducers

DAB assay- The activation of defense responses against biotic and abiotic stresses can be effectively indicated by the accumulation of reactive oxygen species (ROS). The DAB (3,3'-diaminobenzidine) assay utilizes the oxidation of ROS in the presence of heme-containing proteins like peroxidase, resulting in the formation of a dark brown precipitate in stressed plant tissues. As shown in Fig. 2, ROS accumulation was absent in control plants (J), but evident in all treated plants. Notably, the highest level of ROS accumulation, indicated by intense browning of leaves, was observed in treatment T5 (1.5 mM BTH), while the lowest level was observed in treatment T_3 (2.5 mM SA).

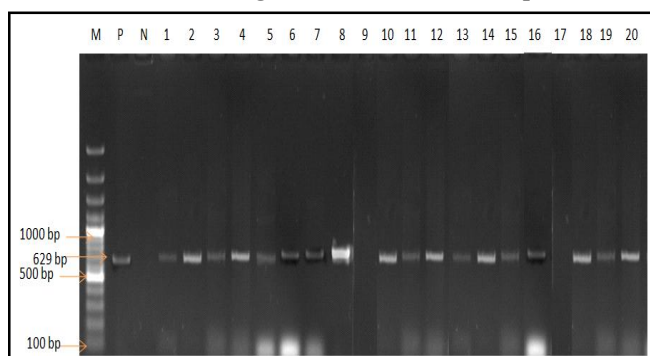


Fig. 1: Gel electrophoresis representation of PLRV in SAR treated plants, M. 100 bp ladder; P. Positive Control; N. Negative Control; 1- 2. T_1 (0.5 Mm SA); 3- 4. T_2 (1.5 Mm SA); 5- 6. T_3 (2.5 Mm SA); 7- 8. T_4 (0.5 Mm BTH); 9- 10. T_5 (1.5 Mm BTH) And 11- 12. T_6 (2.5 Mm BTH); 13- 14. T_7 (0.5 Mm INA); 15- 16. T_8 (1.5 Mm INA); 17- 18. T_9 (2.5 Mm INA) And 19- 20. T_{10} (Control).

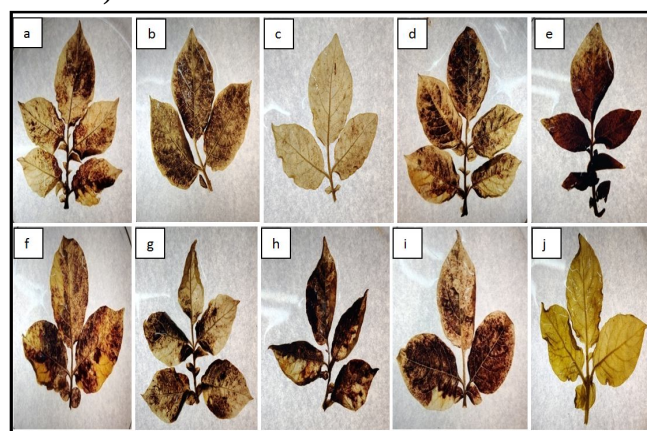


Fig. 2: DAB assay of SAR inducers treated plants after 24 hours, (a) T_1 (0.5 mM SA), (b) T_2 (1.5 mM SA), (c) T_3 (2.5 mM SA), (d) T_4 (0.5 mM BTH), (e) T_5 (1.5 mM BTH), (f) T_6 (2.5 mM BTH), (g) T_7 (0.5 mM INA), (h) T_8 (1.5 mM INA), (i) T_9 (2.5 mM INA) and (j) T_{10} (control).

Table 3: Activity of poly phenol oxidase (PPO) in potato plants at different hours post inoculation with SAR chemicals.

Treat-ments	PPO activity ((Δ) Abs/ min/g)			
	0 hr	24 hrs	48 hrs	72 hrs
T ₁	0.027	0.224 ^{h*}	1.855 ^h	2.033 ^g
T ₂	0.023	0.260 ^f	2.384 ^f	2.616 ^e
T ₃	0.025	0.240 ^g	2.057 ^g	2.152 ^f
T ₄	0.026	0.570 ^e	3.372 ^c	3.656 ^b
T ₅	0.020	0.674 ^a	3.830 ^a	4.342 ^a
T ₆	0.021	0.587 ^b	3.177 ^b	3.284 ^c
T ₇	0.023	0.345 ^e	2.496 ^e	2.674 ^e
T ₈	0.025	0.384 ^d	2.687 ^d	3.012 ^d
T ₉	0.025	0.352 ^e	2.397 ^{ef}	2.632 ^e
T ₁₀	0.026	0.031 ⁱ	0.037 ⁱ	0.042 ^h
SD	0.002	0.196	1.036	1.146
CD (p=0.05)	NS	0.007	0.105	0.086
*Values superscripted with same letters are significant at 5 per cent [T ₁ – 0.5 mM SA; T ₂ – 1.5 mM SA; T ₃ – 2.5 mM SA; T ₄ – 0.5 mM BTH; T ₅ – 1.5 mM BTH; T ₆ – 2.5 mM BTH; T ₇ – 0.5 mM INA; T ₈ – 1.5 mM INA; T ₉ – 2.5 mM INA; T ₁₀ – control]				

The accumulation of Reactive Oxygen Species (ROS) in response to exogenous application of Systemic Acquired Resistance (SAR) inducers serves as a hallmark of defense response activation in plants, conferring protection against both biotic and abiotic stresses. Lee and Huang (2005) investigated the role of oxidative bursts in the establishment of SAR in chilli plants, demonstrating the systemic activation of resistance in leaves distant from the pathogen attack, thereby exhibiting a systemic pattern of resistance activation. Furthermore, Yang *et al.*, (2017) employed the DAB staining assay to visualize the endogenous ROS activation, revealing the activation of disease resistance.

Assay of poly phenol oxidase (PPO) activity in SAR inducers treated potato plants- As shown in Table 3, the activity of polyphenol oxidase (PPO) in plants treated with SAR inducers exhibited a temporal increase. Initially, at 0 hours, PPO activity was negligible, but it rapidly increased following the exogenous application of SAR inducers, as evident from the subsequent time intervals. At 72 hours, the control treatment (T₁₀) showed the lowest PPO activity, with a value of 0.042 (Δ) Abs/min/g. Among the SAR inducer-treated plants, the lowest PPO activity was observed in treatment T₁ (0.5 mM SA), with a value of 2.033 (Δ) Abs/min/g, followed by treatments T₃ (2.5 mM SA) and T₂ (1.5 mM SA), with values of 2.152 (Δ) Abs/min/g and 2.616 (Δ) Abs/min/g, respectively. The highest PPO activity at 72 hours post-

treatment was observed in treatment T₅ (1.5 mM BTH), with a value of 4.342 (Δ) Abs/min/g, followed by treatments T₄ (0.5 mM BTH) and T₆ (2.5 mM BTH), with values of 3.656 (Δ) Abs/min/g and 3.284 (Δ) Abs/min/g, respectively.

Polyphenol oxidase (PPO) plays a crucial role in disease resistance by oxidizing phenolic compounds to quinones, which are more toxic to pathogens. In the present study, the SAR inducer S-Methyl 1,2,3-benzothiadiazole-7-carbothioate (BTH) at a concentration of 1.5 mM was found to be most effective in suppressing Potato Leafroll Virus (PLRV). Consistent with our findings, Karthikeyan *et al.*, (2009) observed elevated PPO activity in urd bean plants treated with SA and BTH, which were infected with Urd Bean Leaf Crinkle Virus (ULCV). Their study revealed that PPO activity peaked on the third day post-treatment and subsequently decreased, with BTH-treated plants exhibiting higher PPO activity compared to SA-treated plants. Our results align with these previous findings, further solidifying the role of PPO in disease resistance.

Conclusion

The presence of Potato Leaf roll Virus (PLRV) in local potato landraces is a significant factor contributing to their degeneration. Given the prevalence of these indigenous varieties in the North Eastern states, addressing viral degeneration is a critical priority. Therefore, the methods described in this paper represent initial efforts towards eliminating PLRV. Notably, among the Systemic Acquired Resistance (SAR) inducers evaluated, BTH at a concentration of 1.5 mM demonstrated the most promising results, achieving an impressive 87.03% reduction in PLRV incidence compared to the control at 30 days post-treatment.

Systemic Acquired Resistance (SAR) inducers, while effective in promoting disease resistance, can also exhibit phytotoxicity at higher concentrations. For instance, salicylic acid (SA) is non-phytotoxic at concentrations below 5 mM, but displays phytotoxic effects at 10 mM (Frey and Carver, 1998; Barilli *et al.*, 2010). Therefore, consideration of these phytotoxic thresholds is crucial when applying SAR inducers for disease management in plants. Given the broad-spectrum protection offered by SAR against various pathogens, it can complement conventional pesticides as a supplementary tool. Moreover, the induction of SAR provides an additional strategy for controlling viral diseases, which can be used in conjunction with genetically resistant lines to prevent crop infection, offering a multi-faceted approach to disease management.

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