



Original article

Enhancement of resistance by poultry manure and plant hormones (salicylic acid & citric acid) against tobacco mosaic virus



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ABSTRACT

Virus is the most menacing factor for plant, which causes enormous economic losses in agriculture worldwide. Tobacco mosaic virus is most hazardous virus among the plants that can spread through biological and non-biological sources. TMV is ancient virus that causes huge economic losses to pepper cucumber ornamental crops and tobacco. It can be controlled by reducing the population of vector through pesticide application. However, the rapid usage of synthetic chemicals causes environmental pollution and destroys our ecosystem. Consequently, different approaches just like natural derivatives should be adopted for the environmental friendly management for TMV. This in vitro study demonstrated the potential role of natural metabolites such as poultry manure and plant extracts such as salicylic acid and citric acid for the control of TMV. Two different concentrations of poultry manure 60G and 30G were used. Poultry manure was mixed with the soil at the time of sowing. Disease severity was minimum at maximum concentration as compared to the control. Meanwhile, two different concentrations of salicylic acid and citric acid 60% and 90% were applied by foliar sprayer after three-leaf stages. Disease severity was observed after 5, 10, 15, 20, 25, and 30 days after disease inoculation. Here also maximum concentration showed the minimum disease severity and higher concentration of both animal and plants extracts were used for following experiment. Quantitative real-time PCR (RT-qPCR) results demonstrated that different plant defense-related genes such as *PR1a*, *PAL*, *PR5*, *NPR1*, *PR1b*, and *PDF1.2* were up-regulated. Furthermore, applications of each treatment-induced systemic resistance against a wide range of pathogen including TMV and fungal pathogen *Botrytis cinerea*.

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1. Introduction

Yield losses in agriculture are mainly due to the attack from pathogens. Naturally, crops growing in a soil, are mainly surrounded by different microorganisms that might be pathogenic or beneficial. Pathogenic microorganisms cause severe disease and also infect the plants. But beneficial bacteria, just like the plant growth-promoting rhizobacteria (PGPR) can decrease the effect of pathogenic microorganisms by antagonistic effect of microbes through competition of nutrients, secretion of lytic enzymes, and production of antibiotics (Handelsman et al., 1996; Van Loon et al., 2003; Van Loon et al., 2004). Moreover, PGPR can decrease

the disease severity and multiplication of pathogens indirectly eliciting the plant defence system (Haas et al., 2005), which produces long-term effect against microorganisms (Diez-Navajas et al., 2008; Pieterse et al., 2012). The disease spectrum promoted by PGPR inducing systemic resistance (ISR) differs enhanced resistance which matches partly pathogenic-induced systemic resistance produced by the association of pathogen molecular patterns (Boller and Felix, 2009). Both systemic acquired resistance (SAR) & ISR showed the state to increase the basic resistance in plant that depends on the signalling pathway jasmonic acid and salicylic acid. These two hormones play a vital role in the regulation of signalling system, which induced defense system (Pieterse and Van Loon, 1999; Glazebrook, 2001). Usually, rhizobacteria induced ISR that is independent of JA but requires SA and ethylene signalling pathway in plants.

Tobacco (*Nicotiana tabacum*) is an important crop belongs to family Solanaceae (Nightshade) and native to the North and South American continents. It is widely cultivated in Pakistan, India, China, America, Indonesia etc. China is the largest country of tobacco production. (Yang and Klessig, 1996). Tobacco mosaic virus (TMV) contains various type of metabolites such as phenol, flavonoids, proteins alkaloids and polysaccharide. From 2001, researcher made progress to identify the products based on different plants microorganisms and animal to control tobacco mosaic virus (Shibuya and Minami, 2001; Li et al. 2007; Miresmailli and Isman, 2014; Zhao et al. 2013; Zhao et al. 2015; Zhao et al. 2015).

Therefore, the effect of viral disease can be minimized by reducing the population of vector and pesticide application (Islam et al. 2017), but the usage of such treatments never completely reduce the viral infection because it does not hit directly upon the virus (Schreinemachers et al. 2015). Moreover, the pesticide badly affects on the human and other ecosystem (Islam et al. 2017; Islam and Ahmed, 2016; Islam et al. 2016; Tayyab et al., 2018; Idrees et al. 2017). Just like dichlorodiphenyltrichloroethane (DDT) and organophosphate which were introduced in 1930s for the control of insect pest, but severely affects the human beings with continuous use of about 10 years (Slootweg et al. 2010; Zhao et al. 2017; Arshad et al. 2016; Noman et al., 2017a, 2017b).

Moreover, the effects of pesticide on human health and environment, researcher change their thoughts in the direction of previous time when people use natural products and herbal extracts for the control of disease. For the control of plant viruses and their management, they also used natural extracts from plant microorganisms and animal (Islam et al. 2017; van Bruggen et al., 2016), furthermore commercialized it as a bio-pesticide (Chandler et al., 2011). The bio-pesticide has minimized residual effects on human being, environmental friendly and host specific. Moreover, it's not susceptible for virus to attain a long resistance (Kumar, 2012; Wang et al. 2012).

However, mostly animal metabolites anti-viral activity against plant have not been studied. That's why it is suggested that couple of oligosaccharide chitosan and chitin anti-viral properties against plant (Jia et al. 2016). These are chitosan-based products that can activate plant defense mechanisms against viruses (Lu et al. 2010). Most probably against tobacco mosaic virus, chitosan has 50.41% inhibition rate at a concentration of 50 µg/mL (Cowan, 1999). Herbal extracts always proved our thoughts on the basis of previously used for the cure of human, animal, and plant disease (Pandey and Rizvi, 2009; Noman et al. 2013; Noman et al., 2017a, 2017b; Jing et al. 2012). But in the early decade's researchers thoughts to find the various plant-based products for anti-viral activity (Elbeshehy et al. 2015; Wang et al. 2015; Choudhary and Sharma, 2014). Later on, their efforts declared that primary plant-based products such as protein and polysaccharides have better results against plant viruses.

The aimed of this study was to investigate the effect of animal metabolites such as poultry manure, plant extracts salicylic acid and citric acid to enhance the resistance against tobacco mosaic virus. As the activity of plant defence signalling mechanism was mediated by these animal and plant extracts at different concentrations.

2. Material and method

2.1. Condition for plant cultivation

Nicotiana tabacum was grown at 24–26 °C with 12–14 h dark/light in a growth chamber with relative humidity 65–70%. Tobacco cells were grown at 25 °C in a murashige and skoog medium (Murashige 4.2 g/L and 100 mg/ml skoog media in 2, 4-dichlorophenoxyacetic acid, inositol, 0.2% KH₂PO₄, 0.2 mg/ml and sucrose 3% and 1 mg/LVB1 having pH 5.2) in shaker at 150 rpm for a night. For Bioassay TMV was grown in tobacco.

2.2. Bioassay with poultry manure and plant extracts (Salicylic acid and citric Acid) induced resistance against tobacco disease.

Different concentration of poultry manure and plants extract was prepared from the stock solution. Poultry manure was collected from the countryside of Guiyang City in the Guizhou provinces of China. Each treatment with two different concentrations was used along with buffer as a control to check the effectiveness of the animal and plant extracts against tobacco mosaic virus. A six-week plant was used to check the disease severity index. Poultry manure with two concentrations (30G and 60G) was mixed with soil, and plant extracts (60% and 90%) were applied on the plant leaves with an aerosol spray bottle unless the tobacco plants were covered thoroughly and became drains. About 150–200 G of poultry manure was mixed in each pot, and about 4–5 ml plant extracts were applied on each plant. Disease severity was measured after 5 days of interval and observed for 30 days. Each treatment was replicated 10 times, and the bioassay was repeated 3 times. After the application of treatments, systemic leaves were inoculated with the infectious clone of TMV. Treated plants were maintained under controlled conditions and disease symptoms were observed daily. The disease severity was observed by counting the TMV lesions and measuring the lesion diameter at 5 days post inoculation. For measuring the lesion diameter, 10 random diameters were observed at each plant. The resistance level induced in tobacco against TMV was determined at 5, 10, 15, 20, 25, and 30 days using 0–5 arbitrary scale. Rating of TMV were as followed 0 for no. of leaves showed no symptoms, 1 for slightly yellowish 1–10% of leaves become yellowish, 2 for 11–25% of leaves become yellowish with curling upward. 3 for scattered yellowing in colour with 26–50%. 4 for prolonged curling up to 50–75%. 5 for interveinal chlorosis showed by systemic leaves dwarf stunted and wrinkled (Sokea et al 2019).

2.3. Disease severity Index:

Disease severity index was measured by the following formula.

$$\frac{\text{Sum of all the disease rating}}{\text{Total no. of plants} \times \text{maximum disease rating}} \times 100$$

2.4. Extraction of RNA and cDNA synthesis:

To check the molecular characterization of poultry manure, salicylic acid and citric acid induced resistance against TMV; plants

Table 1
Primers used for RT- qPCR in this study.

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
PR1a	TAGCGCTAATGCAGCTCTG	GAGTAGCATTACGGTACGA
PAL	GTCATTGCATGCTCACCTGC	AATCGACTCTCGGTAATCGA
PR5	GTCCCATATCGAATGACGA	TACTGTCTGGAATCCTAGCAG
PR1b	AAATGCTTACAATCGATTAC	CCAATGAGGATGACCGTCCA
NPR1	TTGTAGCTGATATGGCTCCA	GAACGTGCAACTATGACGTA
PDF1.2	TCTAGATGGCACATTGAAGC	TGGTACTGACGTATGACGTC
Actin	CCCCTACTAGATCTGACCAT	AATCGAATTGCCGATGGCTA

were treated with best concentration of each treatment after the inoculation of disease. Leaf samples were collected after 1, 2, 3, 4, 5 days post inoculation of the treated and controlled plant and stored at -80 °C until used. RNA was extracted from the leaf samples by using HP total RNA R6812-01 ® Plant RNA Kit (OMEGA

Biotech, Beijing, China) using following the manufacture protocol. The RNA quality was observed by nanophotometer. After extraction, RNA was transcribed in to cDNA by using kit. First-strand cDNA was synthesized using the Revert Aid first stranded cDNA K1622 ® All in One Super Mix for qPCR Kit (Thermo scientific, Beijing, China), followed the protocol of manufacturer and the concentrations of the cDNAs were adjusted to be the same.

2.5. Real time quantitative polymerase chain reaction (RT-qPCR)

To assess the relative expression of resistance genes related TMV of treated and control plant were performed. These resistance genes related to TMV PR1a, PAL, PR5, PR1b, NPR1, and PDF1.2. β-Actin (reference genes) were used as internal control. Primers pairs used for the RT. qPCR amplification were given in the Table 1. RT-qPCR amplifications were performed on thermo cycler ABI 7500

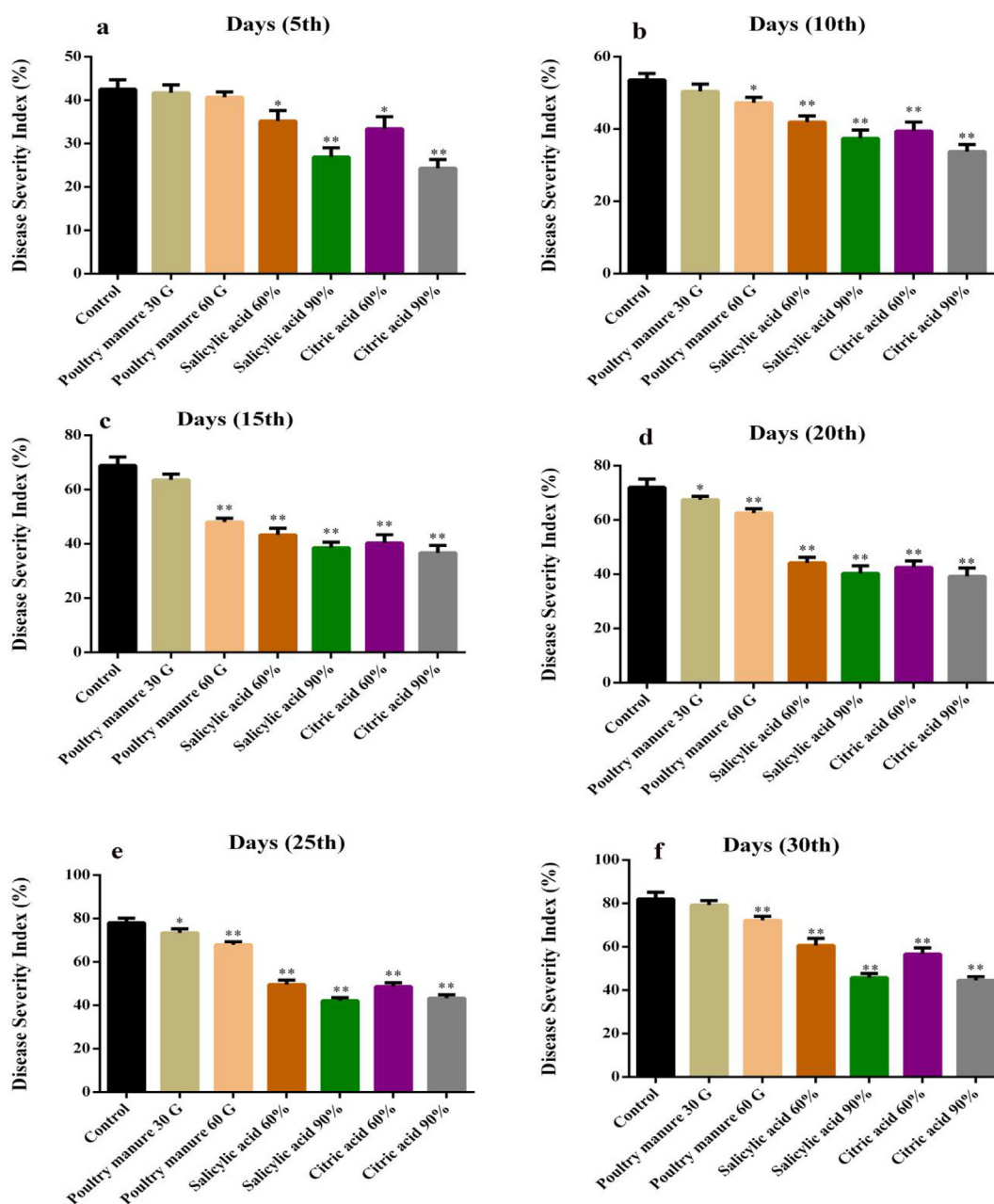


Fig. 1. a-f showed the disease severity after the treatment of poultry manure salicylic acid and citric acid on different concentration different day post inoculation. Asterisk symbol showed the significant difference between the concentration of treatments and control by using student *t*-test.

Real-Time PCR System (Applied Biosystems, USA) using 20 μL of reaction mixture (comprising of 10 μL of $2 \times \text{SYBR}^{\text{®}}$ Premix Ex Taq (Takara, Dalian, China), 0.5 μL of each 10 $\mu\text{mol L}^{-1}$ forward and reverse primers, 2 μL of 10-fold diluted cDNA template and 7 μL of ddH_2O). Thermal protocol included preheating at 95 $^{\circ}\text{C}$ for 30 s, followed by 40 cycles of denaturation at 95 $^{\circ}\text{C}$ for 30 s and annealing at 60 $^{\circ}\text{C}$ for 40 s and elongation at 72 $^{\circ}\text{C}$ for 60 s. There were three biological replicates for each treatment and three technical replicates for each sample.

2.6. Data analysis:

The experiment was repeated three times. Data were presented as the mean and standard deviation. The statistical analysis was performed using R Version 3.5.0 (www.R-project.org). The aov function was used to calculate the analysis of variance (ANOVA) in R version 3.5.0. By using student *t*-test significant differences between treatment and control were determined by using R software. The gene expressions were obtained by CT ($2^{-\Delta\Delta\text{CT}}$) method. Significant level was measured with student *t*-test at $\alpha = 0.05$ was used for comparing fold change in both treated and control samples.

3. Results

3.1. Effect of poultry manure, salicylic acid and citric acid against TMV

The data were normally distributed, and analysis of variance (ANOVA) showed significance difference in treatments. Further, student *t*-test exhibited a significant difference between the treatments and concentration for disease severity. At higher concentration (60G) of poultry manure minimum disease severity was found at 5th day after post inoculation (40.7) and maximum was found at

30th day after post inoculation (72.23) as the control. The significant difference was found between the control and treated plants showed in Fig. 1. While on the other hand at lower concentration 30G of poultry manure the minimum disease severity was found at the 5th day after post inoculation and maximum was found at 30th day after post inoculation. There is no significant difference found between the control and treatment of poultry manure after 5th, 10th, 15th, 20th, and 30th day post inoculation shown in Fig. 1. That's why 60G poultry manure was considered as best concentration and was used in the follow-up work. On other hand similar trends were found by using plant extracts salicylic acid and citric acid. The minimum trends were found at after 5th day post inoculation and maximum disease severity were found at 30th day after post inoculation. In case of plant hormones, the maximum effect was found at higher concentration 90% while minimum was found at lower concentration 60% as compared to the control. Significant difference was found between the treated and control plants. Heat map showed that salicylic acid and citric acid 60% were found in a same zone. While other all concentration were at different zone shown in the Fig. 2.

3.2. Relative gene expression of resistance gene after the application of poultry manure, salicylic acid and citric acid

Poultry manure enhances the defence response due to tobacco mosaic virus and induced defence response in a tobacco. PR1a, PAL, PR5, PR1b, NPR1 and PDF1.2 were chosen as the marker genes which induced resistance against this viral disease. After the application of poultry manure 60G, all genes were up-regulated. After each day of post inoculation except one genes NPR1 genes was down regulated after 5th day of post inoculation. The maximum expression level was found at 3rd and 4th day after post inoculation. The expression levels were gradually increased with the

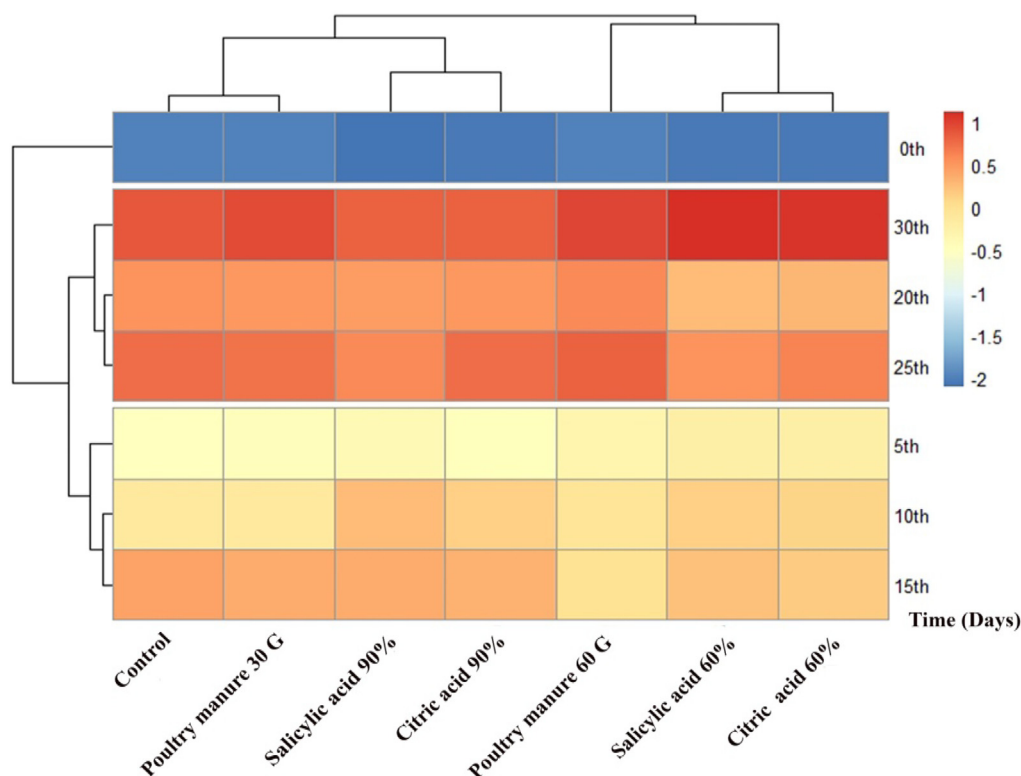


Fig. 2. Heat map analysis of different treatments at different concentrations. On X-axis showed the concentration of treatments, while on y-axis showed the day post-inoculation. Heat map was obtained by using heatmap.2 in R Version 3.5.0 (www.R-project.org). Different colours displayed the effects on disease.

extension of days but after 4th day post inoculation it was gradually reduced (Fig. 3a) and similar trends were found after the application of salicylic acid 90%. All genes were up regulated at each interval of time except PR5 and PDF1.2. PR5 was down regulated after 5 days post inoculation, while PDF 1.2 was down regulated after 1st and 5th day post inoculation. NPR1 showed no difference after 1st day inoculation (Fig. 3b). Different trends were found after the application of citric acid. PR1a and NPR1 after 5th day post inoculation showed no difference with control, while PAL and PDF1.2 showed down regulated after 1st day of post inoculation. While the other genes were up-regulated at each interval of time (Fig. 3c). But the similar trends were found at each treatment maximum up-regulation was found at 3rd and 4th day after post inoculation of disease.

4. Discussion

Animal metabolites and plant extracts play major to enhance disease resistance and biological control of plant disease. Mostly animal metabolites with anti-virus activities against plant diseases have been identified (Wink, 2015). This study conducted in vitro animal metabolites such as poultry manure to determine its potential role against TMV. According to the result, disease severity

index decreased with the application of poultry manure as compared to the control treatment. Our results were in agreement with the previous study in which the animal metabolites such as chitosan concentration effect the inhibition rate of the tobacco mosaic virus disease (Islam et al 2018). Furthermore, it is demonstrated that how animal metabolites such as couple of oligosaccharide chitosan and chitin have anti-virus activity against plant virus (Wink, 2015). Our result confirmed that chitosan have inhibitory effect are regulated via hydrogen peroxide, nitric oxide, and protein kinase activity which regulated the plant signalling pathway, which was consistent with previous studies (Handelsman et al., 1996; Van Loon et al., 2003; Diez-Navajas et al., 2008).

Plant extracts also play a vital role on the basis of historical uses of herbal medicine and herbs for curing of human animal and all type of plant diseases (Wang et al 2015, Choudhary and Sharma, 2014; Sokea et al 2019; Wink, 2015). Moreover, plant secondary metabolites such as alkaloids, phenolics, flavonoids, and cretanin play a major role in biotic and abiotic stresses which are mostly used as anti-fungal antibacterial and anti-viral disease. Some of the primary metabolites are being actively used to enhance TMV (Sokea et al 2019, Islam et al 2018). Our results were in accordance with the previous result in plant secondary metabolites were used against the plant viral disease such as TMV. Some microorganisms

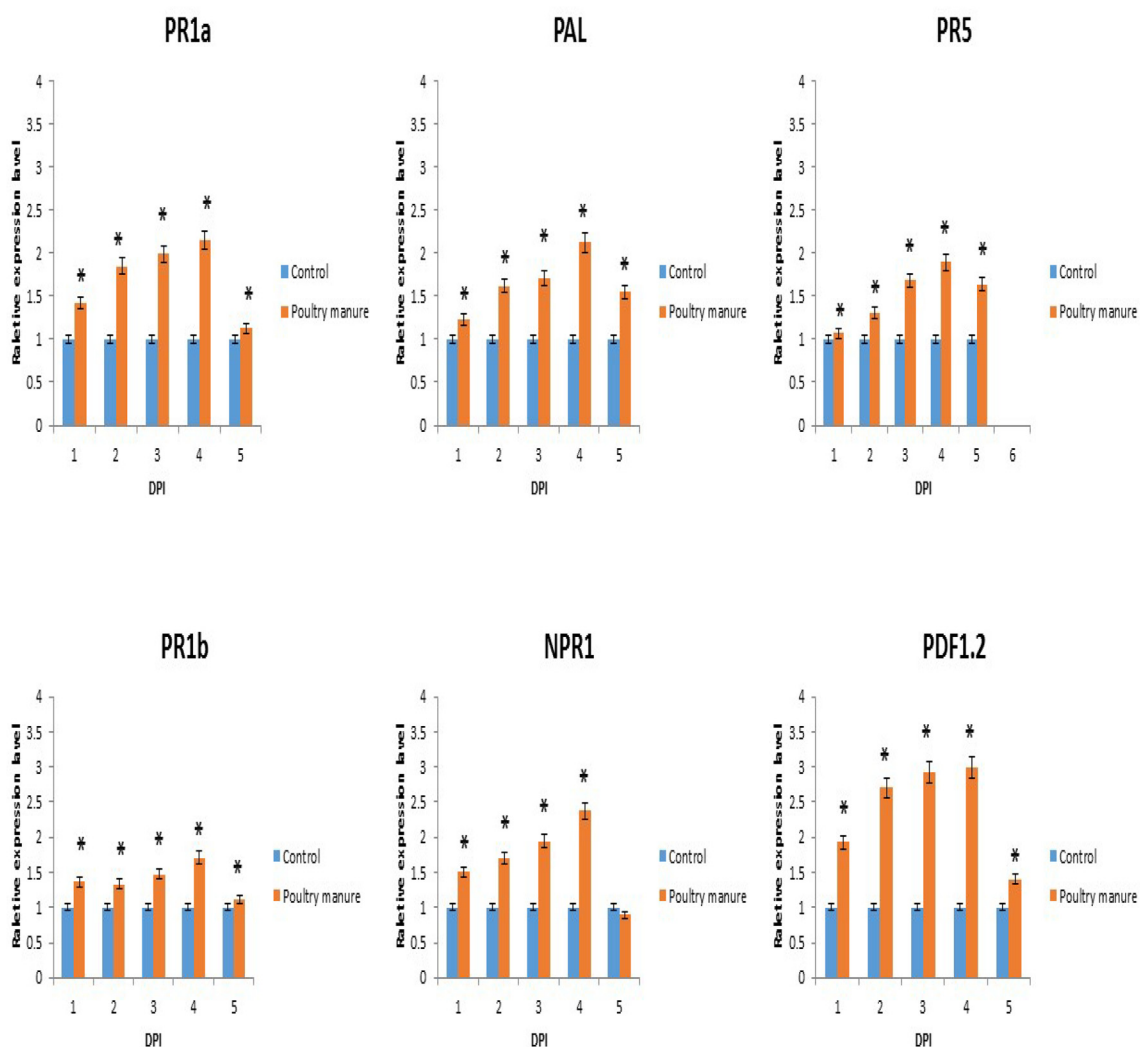


Fig. 3a. Showed the result of relative expression level of gene expression of different defence related genes of tobacco after the treatment of poultry manure. The sample was collected from systemic leaves at different time interval. Orange bar show the treated sample, while the blue bar showed the control treatment. Asterick symbol showed the significant difference between the concentration of treatments and control by using student *t*-test.

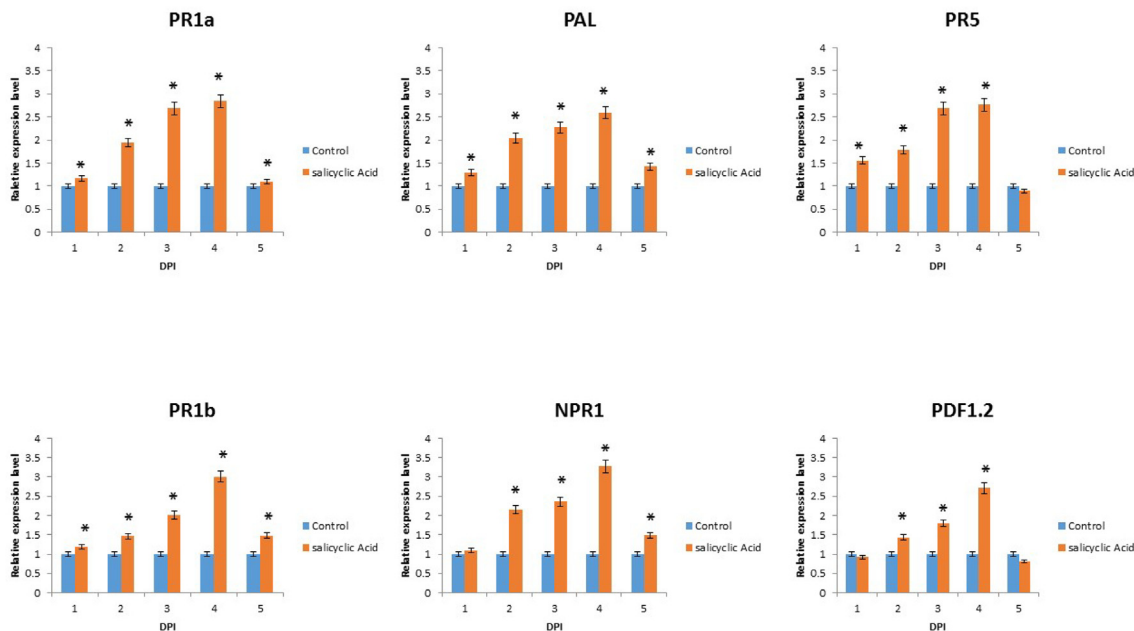


Fig. 3b. Showed the result of relative expression level of gene expression of different defence related genes of tobacco after the treatment of salicylic acid. The sample was collected from systemic leaves at different time interval. Orange bar show the treated sample, while blue bar showed the control treatment. Asterick symbol showed the significance difference between the concentration of treatments and control by using student *t*-test.

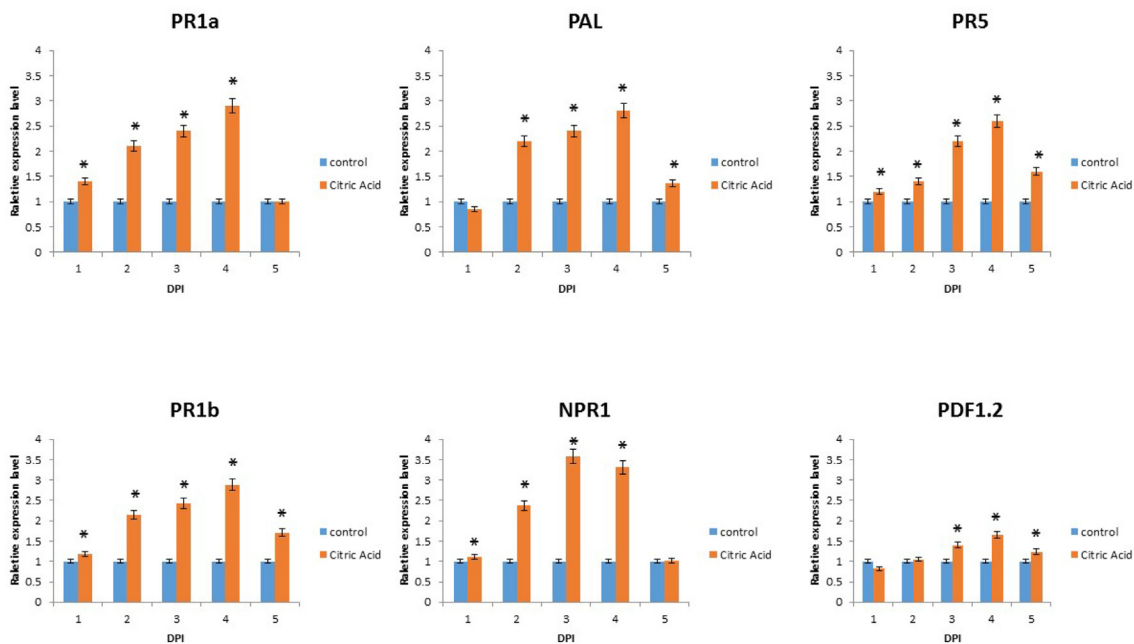


Fig. 3c. Showed the result of relative expression level of gene expression of different defence related genes of tobacco after the treatment of Citric Acid. The sample was collected from systemic leaves at different time interval. Orange bar show the treated sample while blue bar showed the control treatment. Asterick symbol showed the significance difference between concentration of treatments and control by using student *t*-test.

such as actinomycetes, bacteria and fungi are also used for the control of plant disease (references). Different elicitors were extracted from microorganisms were used against plant diseases such as TMV and tomato yellow leaf curl virus which also enhanced the plant signalling pathway (Yasuhara-Bell and Lu, 2010). Our study was also in accordance with the previous study (Tayyab et al 2018-Idrees et al 2017).

Animal and plant secondary metabolites enhance resistance of various pathogens, including TMV (Wang et al., 2016). Different plant and animal secondary metabolites which induced systemic

resistance which are controlled by plant signalling pathway in which plant hormones such as JA, SA and ethylene play a vital role (Zhao et al 2007). Our study provides evidence in which poultry manure, salicylic acid and citric acid enhance expression of various SA responsive genes such as *PR1a*, *PAL*, *PR1b*, *PR5*, and *JA* responsive genes, including *PDF1.2* (Fig. 4). Moreover, poultry manure and plant extracts such as salicylic acid and citric acid enhance the expression of *NPR1*, which plays an important role to induce the PR genes with the interaction of TGA transcription factor (Kinkema et al 2000, Kim and Delaney, 2002, Zhou et al 2000).

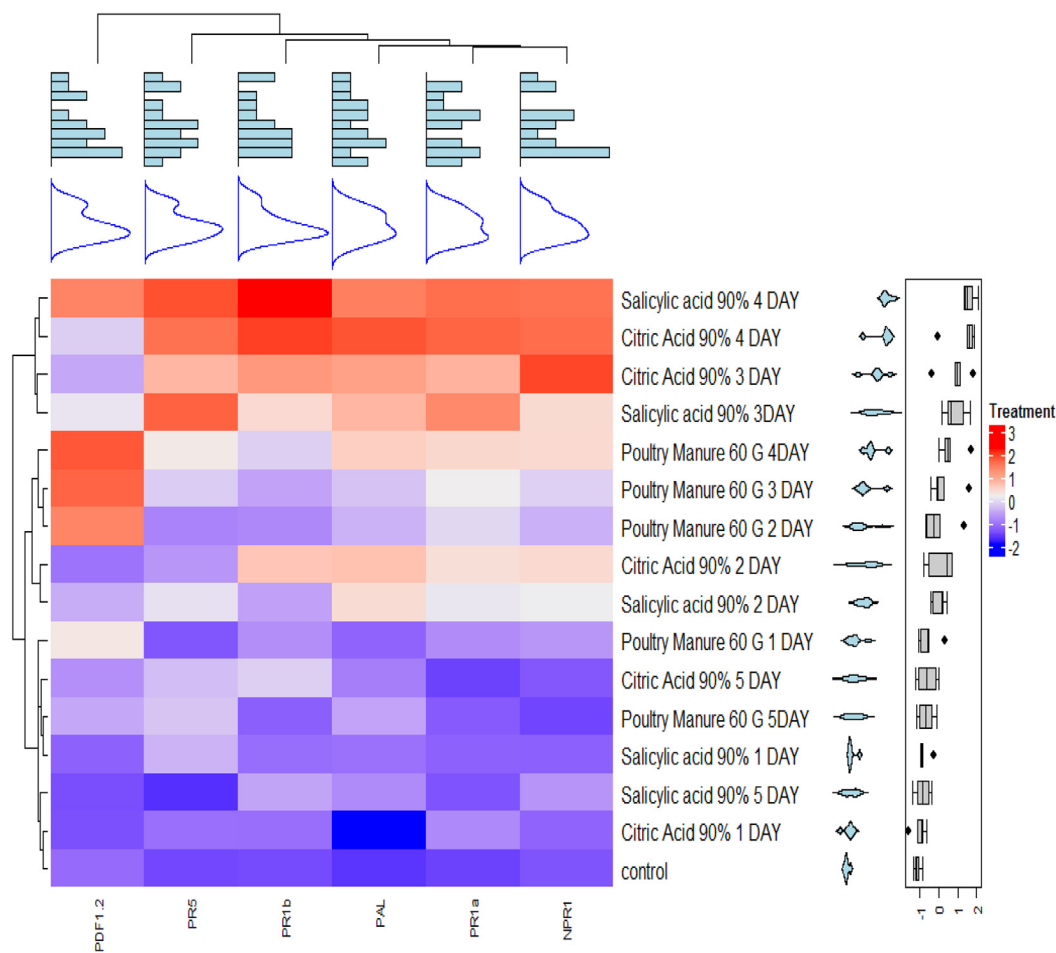


Fig. 4. Heat map of the gene expression data at different concentration of treatments. On X-axis showed the name of genes, while on y-axis showed the different concentrations of treatments. Heat map was obtained by using heatmap.2 in R Version 3.5.0 (www.R-project.org). Different colours displayed the expression level.

These result described that SA and JA plant signaling pathway genes involved in the animal and plant extracts enhanced systemic resistance in tobacco against TMV. Furthermore, accumulation process of secondary metabolites which acted as plant defence infection producing mechanical barrier against the growth of pathogen. Phenolic compound contains phenolic acid and scopoletin and cell wall combined the ferulic acid (Chaerle et al 2007). PAL (phenylalanine aminolayse) is a major enzyme for the production of different phenolic compounds in plant signalling pathway (Hano et al 2008). Respectively RT-qPCR analysis showed that PAL genes were induced by poultry manure and salicylic acid and citric acid in tobacco.

5. Conclusion

In spite of the fact that plant viral disease results in enormous economic losses in the world, in summary we hypothesized that poultry manure and plant extracts such as salicylic acid and citric acid induced resistance against TMV. Through a series of experiments we described that plants treated with poultry manure and plants extracts at different concentration showed minimum disease severity as compared to the control plants. Furthermore, molecular mechanism of defence related genes potentially involved in SA and JA pathway demonstrated that the application of animal and plant extracts induced a significant expression level of the resistance genes in tobacco against TMV. The result of our

study demonstrated that such natural products could be used as novel biological tools against plant virus disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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