RESEARCH | Egyptian Journal of Agricultural Research | Plant Pathology

Molecular identification of *Alfalfa Mosaic Virus* and utilization of lactoferrin against viral infection in periwinkle plants

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Received: 25-03-2024; **Accepted**: 23-05-2024;**Published:** 01-06-2024 **DOI:** 10.21608/EJAR.2024.279298.1531

ABSTRACT

Alfalfa mosaic virus (AMV) was isolated from naturally infected periwinkle plants obtained from Giza Governorate. Characteristic symptoms, including yellow blotches, vein banding, and chlorosis, were observed. Through the isolation of single local lesions, the virus was purified biologically using *Chenopodium amaranticolor* Coste & Reyn. Identification of the virus was conducted by employing various methods, including symptomatology, transmissibility, and serological assays such as DAS ELISA, electron microscopy, and molecular detection. lactoferrin (LF) at different concentrations (1000, 750, 500, 250, and 100 mg/L) was used as a resistance inducer to mitigate *alfalfa mosaic virus* infection. The highest concentration of lactoferrin (LF), applied five days prior to inoculation, resulted in a 90% reduction in viral infection. All lactoferrin concentrations led to significant increases in phenol levels and enzyme activity. Treatment with different concentrations of LF resulted in the induction of new proteins.The maximum levels of vinblastine and vincristine were detected at the highest lactoferrin concentration, reaching 0.393 and 0.399 respectively, five days post inoculation. Lactoferrin treatments also increased the concentrations of photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoids.

Keywords: *Alfalfa mosaic virus* (AMV), Identification, Lactoferrin, Induce resistance, Protein and Enzyme analysis

INTRODUCTION

Periwinkle (*Catharanthus roseus* (L.) (G.Don) stands out as one of the most significant medicinal plants within the *Apocynaceae* family. Traditional medicine is specific for treating diabetes, with historical documentation indicating that a leaf extract has leukopenic effects on mice (Mohamed *et al*., 1987). Among its notable constituents are four primary vinca alkaloids, namely, vincristine, vinblastine, vindesine, and vinorelbine, which are applied in diverse cancer chemotherapies (Moudi *et al*., 2013). Vincristine and vinblastine, known for their antineoplastic properties. Correia, (2001), demonstrate potent inhibitory effects against a spectrum of malignancies, including lymphocytic leukemia, solid sarcoma, breast, liver, ovarian, head and neck, testicular, and malignant melanoma (Zhang *et al*., 2018).Alfalfa mosaic virus (AMV) is considered as an important plant pathogen due to its expanded host range. With the ability to infect more than 430 species across 51 dicotyledonous families, AMV poses a considerable threat to agricultural productivity (Jaspars and Bos, 1980). This virus is known to naturally infect numerous herbaceous and certain woody plant hosts, encompassing approximately 150 species within 22 families. *Alfalfa mosaic virus* has a broad host spectrum and are particularly noteworthy in agricultural contexts.

Periwinkle serves as a secondary host for *Alfalfa mosaic virus* (AMV) and acts as a reservoir for the virus when the primary crop is absent. In Egypt, AMV was initially detected and confirmed serologically in broad bean and basil (El-Attar *et al*., 1971 and Shafie *et al*., 1997), and recently, it was molecularly identified in basil, pepper and potato, tomato, alfalfa and clover plants (El-Attar *et al*., 2019, El-Ganainy *et al*., 2023 and Amin *et al*., 2023) respectively. Symptoms induced by the virus include distinct bright yellow blotches accompanied by vein banding and chlorosis, ultimately leading to bronzing of the leaves. The virus targets phloem tissue throughout the plant, including the roots, results in tissue aging and eventual necrosis, often culminating in the death of the infected plant. Transmission of the virus occurs in nonpersistent manner through aphid vectors, seed transmission, and mechanical inoculation via sap (Wintermantel and Natwick, 2012). The architecture of AMV consists of icosahedral particles measuring 30-75 nm in length and 18 nm in diameter. The detection of the virus was achieved using specific primers designed to amplify a conserved region within the coat protein (CP) gene, resulting in the amplification of a 700 bp fragment of the AMV coat protein in infected samples (Al-Saleh and Amer, 2013).

lactoferrin (LF), an iron-binding glycoprotein belonging to the transferrin family, has a molecular weight of approximately 80 kDa. Widely distributed in mammalian secretions and neutrophils, lactoferrin, a key regulator of the immune system, is present in varying concentrations across different biological fluids. Its levels range from 7 g/L in colostrum (first milk) to 1 g/L in mature milk, with the highest concentrations typically observed in human colostrum, followed by human milk and then cow milk (Sanchez *et al*., 1992). Lactoferrin (LF) has diverse biological activities, including antibacterial, antiviral, antifungal, antiparasitic, anticarcinogenic, anti-inflammatory, and antitumor properties (Taha *et al*., 2010; Conneely, 2001; Florian *et al*., 2012).

Some of the effects of lactoferrin stem from its ability to sequester iron, while others result from its interactions with molecular and cellular components of both the host and pathogens. In various transgenic plant systems, such as tobacco, potato, tomato, maize, barley, and ginseng, lactoferrin (LF) has a broad spectrum of biological and preventive activities, including binding, immunomodulation, antibacterial, antifungal, and antiviral properties. It can also neutralize certain bioactive substances, such as lipopolysaccharide (LPS) and glycosaminoglycan. LF is utilized as a recombinant protein for various purposes and has been used as an antiviral agent against plant diseases, such as tobacco mosaic virus in tobacco seedlings and tomato yellow leaf curl virus. The aims of this study were to molecular identification of *Alfalfa mosaic virus* and investigate whether native lactoferrin (LF) could protect against *Alfalfa mosaic virus* (AMV), a significant pathogen that causes severe damage to crops and periwinkle plants in Egypt, by applying lactoferrin aqueous solution to plants in a greenhouse setting.

MATERIAL AND METHODS

Virus isolation and propagation:

Twenty samples were obtained from naturally afflicted periwinkle plants exhibiting characteristic symptoms akin to *Alfalfa mosaic virus* (AMV) infection, including vibrant yellow blotches, vein banding, and chlorosis. These samples were procured from El-Mansoria at Giza Governorate. Subsequently, the plants were relocated and cultivated within a greenhouse environment, each housed in a 25 cm plastic pot filled with natural soil contains sand, peat moss and vermiculite (1:1:1). These plants served as the basis for subsequent procedures involving virus isolation, identification, and serological testing. AMV was then introduced into healthy periwinkle plants in the greenhouse via mechanical transmission from naturally infected plants. The virus was further propagated by mechanically transmitting it to periwinkle plants, which acted as a source for virus propagation. This propagation process involved successive biological purification through three passages on the local lesion host, *Chenopodium amaranticolor* Cost & Reyn (Kuhn, 1964). Additionally, a direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Loewe Biochemica, Germany), was employed for the identification of the isolate under scrutiny.

RNA extraction and RT–PCR amplification of AMV:

Total RNA was extracted from the leaves of healthy, naturally infected, and mechanically inoculated periwinkle plants using the GeneJet™ Plant RNA Purification Micro Kit from Thermo Scientific, USA, following the user manual guidelines. The Verso™ 1-Step RT–PCR Kit from Thermo Fisher Scientific Co. (Waltham, MA, USA) was utilized in accordance with the manufacturer's instructions. Oligonucleotide primers designed by Martinez-Priego *et al*., (2004), namely, AMV coat-F (5'-GT GGT GGG AAA GCT GGT AAA-3') and AMV coat-R (5'-CAC CCA GTG GTC AGC ATT-3'), were used to amplify the conserved region in the CP gene of AMV The RT–PCR mixture was optimized, and a total volume of 25 µl, including 3 µl of RNA, was used for the process. The reaction mixture consisted of 2x one-step PCR master mix, 3.0 µl of 10 µM each primer, 0.5 µl of Verso enzyme mix, 1.25 µl of RT Enhancer, and 4.75 µl of nuclease-free water. The amplification of samples followed specific cycle parameters: an initial RT step at 50 °C for 15 min, followed by the first PCR cycle at 94 °C for 2 min, and 35 cycles, each comprising 30 sec at 94 °C, 30 sec at 54 °C, and 30 sec at 72 °C. A final extension was performed at 72 °C for 10 min. RT–PCR products were separated on a 1% agarose gel, stained with GelStar (Lonza, USA), and visualized under UV light using a Gel Doc 2000 (Bio-Rad, USA). A 100 bp DNA ladder (Invitrogen, CA, USA) was used for size reference.

Electron microscopy:

According to the protocol outlined by Lin *et al*., (1977), a sample was subjected to the leaf-dip preparation method with 2% phosphotungstic acid for negative staining. Subsequently, the prepared sample was examined using a transmission electron microscope (JEOL JEM-1400 TEM, Japan) at the Faculty of Agriculture, Research Park (FARP), Cairo University. Electron micrographs were captured at a magnification of 150,000×.

The effect of lactoferrin foliar application on virus infectivity:

A randomized complete block design was used with three replicates. Each experimental unit had ten pots, with two plants in each pot. Under greenhouse conditions, periwinkle plants were utilized to assess the inhibitory effects of lactoferrin (LF) on Alfalfa mosaic virus (AMV) at concentrations of 1000, 750, 500, 250, and 100 mg/L.

The plants were cultivated individually in plastic pots measuring 40×40 cm, receiving natural sunlight and maintaining temperatures of approximately 23±2 °C. Three groups of seedlings were established for experimentation.

In the first group, the seedlings were treated with lactoferrin (LF) and subsequently inoculated with AMV (1 ml of fresh leaf sap per plant) after 1, 3, 5, and 7 days. Conversely, in the second group, the plants were initially inoculated with AMV and then treated with lactoferrin (LF) (10 ml per plant), after7 days. The third group, which served as the control, consisted of subgroups: C1 - seedlings exclusively inoculated with AMV (infected control); C2 - untreated healthy seedlings (healthy control); and C3 - healthy seedlings treated with lactoferrin (LF) only. Symptoms were monitored regularly, and the inhibitory effect of lactoferrin (LF) was determined using the equation described by Devi *et al.*, (2004): Inhibition% = (A - B/A) × 100, where A represents the number of plants in the control experiment and B represents the number of treated inoculated plants. ELISA tests were conducted in all patients to verify the results.

Protein analysis:

Protein was extracted from lactoferrin-treated periwinkle plants inoculated with alfalfa mosaic virus (AMV) seven days post inoculation, as were similar samples from healthy and AMV-infected plants of equivalent age. Total soluble proteins were extracted from 1 g of each sample following the protocol outlined by Sambrook *et al*., (1989). Proteins were then suspended in loading buffer according to the method described by Laemmli (1970) and subjected to electrophoresis (SDS–PAGE) for 5 hours at 100 volts. Following electrophoresis, the proteins were stained with silver nitrate following the procedure outlined by Sammons *et al*., (1981). The molecular weights of the proteins were determined using a molecular weight standard ranging from 6.5 to 270 kDa.

Total phenol content and enzyme activity:

LF-treated periwinkle plant samples, inoculated control and treated and untreated uninoculated control were collected at three distinct time points (7, 14, and 21 days). Phenolic compound levels in the plants were quantified (mg/100 g plant material) using the method outlined by Maliak and Singh (1980), Ten grams of fresh leaf samples were utilized for each replicate of every treatment to determine the phenolic compounds. Four replicates were used in each treatment. For enzyme activity determination, two grams of fresh leaf weight was allocated per replicate. Four replicates were used for each treatment. Peroxidase activity was identified spectrophotometrically by measuring the oxidation of pyrogallol in the presence of H_2O_2 at 470 nm following the protocol described by Maxwell and Bateman (1967). The polyphenol oxidase enzymes's activity was determined using the method described by Galeazzi *et al*., (1981).

Assessment of Chlorophyll a ,b and carotenoid content (mg/g FW) in Periwinkle leaves in response to viral infection:

The methodologies outlined by Saric *et al*., (1967) were employed to quantify the chlorophyll a and b and carotenoid content in the periwinkle leaves. For each treatment replicate, two grams of fresh leaf samples were used. For each treatment, four replicates were used. Fresh samples weighing 2gm. of the leaf were homogenized using 85%v/v acetone with a little amount of silica quartz and Na_2CO_3 and filtered through a central glass funnel (G4). The residue was repeatedly washed with acetone until it loses its color. The combined extract was finished to a predetermined amount -250 milliliters. For the colorimetric measurement of carotenoids, chlorophyll a, and b at wavelengths of 660, 640 and 440nm, respectively, a sufficient volume was taken. Acetone (80%v/v) was used as a blank in the determination. The following formula was used to compute the pigment contents:

Chlorophyll a (mg/l) = 9.784×E 660-0.99×E640

Chlorophyll b (mg/l) = 21.426× E 640-4.65×E660

Carotenoids (mg/l) = 4.695× E440-0.268×(chl a+b)

Where :E = reading of sample.

The determination of vinblastine and vincristine content was conducted following the procedures described by Nagaraja *et al*., (2002). Five grams of dried leaf sample were used, for each treatment, four replicates were used. Each of a set of 25 ml calibrated flasks in the series received a 3.0 volume of 0.2% dapsone solution. Following an ice bath to cool the flasks, 1.0 milliliter of 0.5% aqueous sodium nitrite was added. Following a 5-minute cooling period, 3.0 milliliters of aqueous sodium acid were added, and the reaction mixture was cooled for an additional 5-minute while being periodically shaked. Within each flask, aliquots of standard vinblastine (12.5-600µg) and vincristine (12.5-300µg) solutions were added, and the flasks were heated in a boiling water bath for 5 minutes. After cooling the reaction mixture in each flask and adding water, the flasks were thoroughly shaken. Vinblastine and vincristine absorbance values were measured at 430nm. in order to quantify yellow-colored azo-product that formed in comparison to colorless reagent blanks. Vinblastine and vincristine calibration graphs were created.

Phylogenetic analysis:

The acquired sequence data of the Coat Protein (CP) gene were subjected to a BLAST search for comparison with 32 reference AMV isolates available in GenBank. The evolutionary relationship was determined using the neighbor-joining analysis method within Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) software, employing bootstrap analysis with 1000 replicates. This analysis, which included pairwise sequence alignment and identity calculation, was conducted to classify the virus, as outlined by Muhire *et al*., (2014) and Tamura *et al*., (2021).

Data analysis:

Two-way analysis of variance (ANOVA) was used with Stat Graphics Centurion XVI (Stat Point Technologies, Inc.) to determine the mean value. Statistical significance was determined by an LSD value of 0.05. CoStat (Cohort Software, USA) version 6.4 was the statistical analysis tool used to analyze all of the data.

RESULTS

Virus isolation and propagation:

The virus under study was isolated from periwinkle plants collected from Giza governorate, Egypt. Compared with those from healthy periwinkle plants, the virus isolated from naturally infected periwinkle plants showed yellow blotches, vein banding and chlorosis on the leaves. AMV was mechanically transmitted from naturally infected periwinkle plants to healthy periwinkle ones grown in the greenhouse (**Fig. 1A, B, & C**). Symptoms such as yellow blotches, vein banding and chlorosis appeared 21 days after virus inoculation. Using single local lesions; the virus biologically purified on *Ch. amaranticolor* Coste & Reyn and was propagated in periwinkle plants that served as the source of the virus. On the other hand, serological identification of AMV by DAS-ELISA using antiserum specific to AMV verified the presence of AMV in both naturally infected periwinkle plants and mechanically inoculated periwinkle plants.

Transmission electron microscopy analysis of the virus using dip preparations from infected periwinkle leaves negatively stained with 2% phosphotungetic acid revealed bacilliform particles with a diameter of approximately 56 nm in length and 18 nm in width (**Fig.1D**).

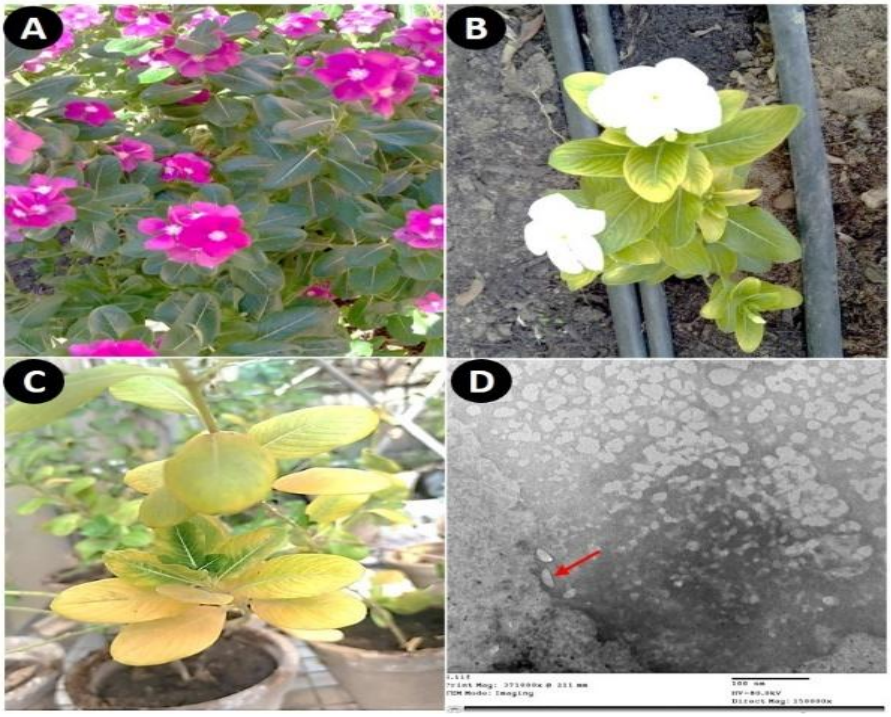

Fig. 1. Periwinkle plant and virus symptoms: (A) representing healthy plants with pink flowers; (B) exhibiting symptoms of vein banding and chlorosis as a result of natural infection; (C) displaying symptoms of yellow blotches, and chlorosis as a result of mechanical inoculation; and (D) showing a transmission electron microscope image at 80,000x magnification of a leaf dip preparation stained with 2% phosphotungstic acid, revealing bacilliform particles in periwinkle plants that are systemically infected

RT–PCR analysis

AMV infection was examined by analyzing RNA extracted from periwinkle plants that were naturally infected or mechanically inoculated. RT–PCR was conducted using the primers AMV coat protein-F and AMV coat protein -R to amplify the conserved region within the AMV CP gene. Agarose gel electrophoresis of the RT–PCR products confirmed the presence of amplified fragments of the expected size, approximately 700bp (**Fig. 2**). Conversely, RNA extracted from uninfected periwinkle plants did not yield any amplified fragments.

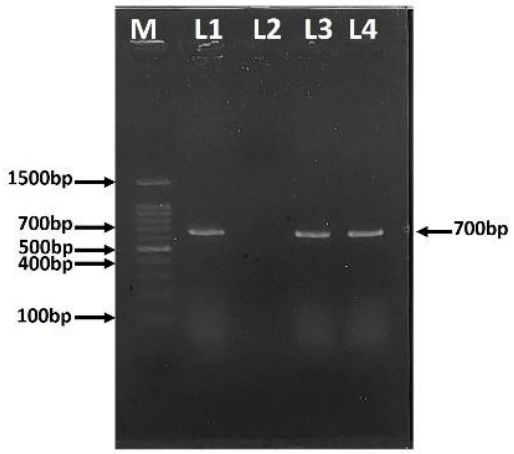

Fig. 2. The results of RT–PCR amplification were analyzed through agarose gel electrophoresis. The gel included a 100 bp DNA ladder as a molecular weight marker. Lane L1 represents a periwinkle sample naturally infected with AMV, while Lane L2 serves as the healthy plant control. Lanes L3 and L4 depict periwinkle samples mechanically inoculated with AMV.

Effect of lactoferrin pretreatment on AMV inhibition under greenhouse conditions:

The effect of lactoferrin pretreatment on AMV inhibition under greenhouse conditions was assessed, as shown in **Table (1) and Fig. (1**). Lactoferrin at different concentrations (100, 250, 500, 750, and 1000 mg/L) was applied, and the inhibition of AMV infection was measured over time (1, 3, and 5 days). The results showed that higher concentrations of lactoferrin led to greater significant inhibition of AMV infection. For instance, at the highest concentration of 1000 mg/L, the inhibition rates reached 75%, 80%, and 90% on days 1, 3, and 5, respectively. Conversely, the inoculated control group, which received no lactoferrin treatment, exhibited substantial AMV infection, with minimal inhibition observed over the observation period. The untreated uninoculated control group showed no signs of infection, as expected. The least significant difference (L.S.D.) values calculated at the 0.05 significance level were used to evaluate the reliability of the observed differences, indicating the precision of the experimental outcome.

Table 1. Effect of lactoferrin pretreatment on AMV inhibition under greenhouse conditions.

Lactoferrin conc. (mg/ L)	Healthy			Infected			Inhibition (%)		
	Days								
	1	3	5	1	3	5	1	3	5
100	5^{d}	7^{d}	9^{d}	15^{b}	13^{b}	11^{c}	25^{d}	35^{d}	45^{c}
250	10^{c}	12^{c}	14^{c}	10^{c}	8^{c}	6^{c}	50^{c}	60^{c}	70^{b}
500	11^{c}	13^{c}	15^{bc}	9^{c}	7^{cd}	5^{d}	55^{bc}	65^{bc}	75^{b}
750	12^{c}	14^{bc}	17^{abc}	8^{cd}	6^{cd}	3^{d}	60^{b}	70^{b}	85^{a}
1000	15^{b}	16^{b}	18^{ab}	5^{d}	4^{d}	2^{a}	75^{a}	80^{a}	90^{a}
Inoculated control	0^{e}	0^{e}	0^{e}	20^{a}	20^{a}	20^{a}	0^{e}	0^{e}	0^{d}
Untreated uninoculated control	20^{a}	20^{a}	20^{a}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{d}
Treated uninoculated control	20^{a}	20^{a}	20^{a}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{d}
L.S.D at 0.05	2.18	2.73	3.37	3.43	3.34	1.52	7.89	6.67	9.43

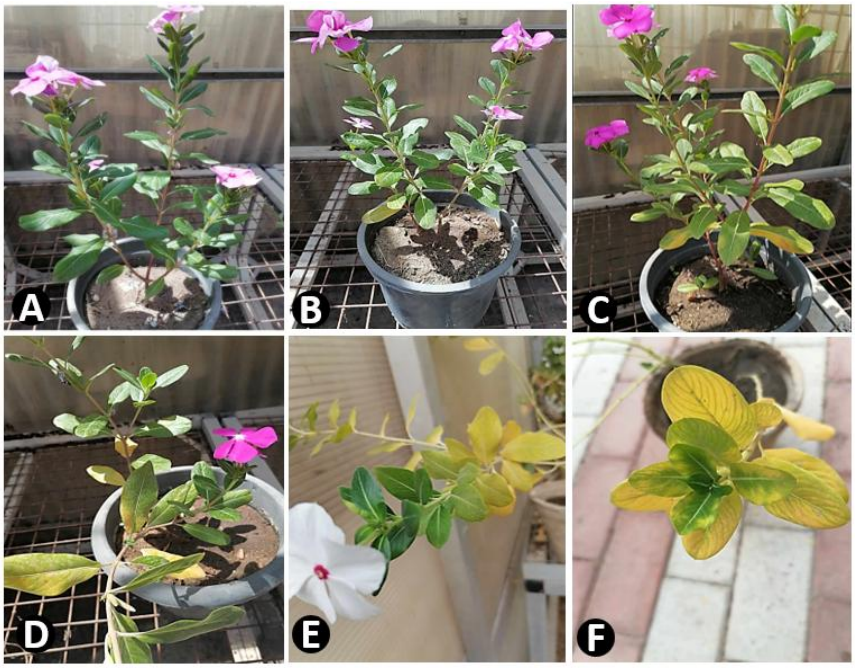

Fig. 3. Periwinkle plants treated with different lactoferrin (LF) concentrations: (A) 1000 mg/L; (B) 750 mg/L; (C) 500 mg/L; (D) 250 mg/L; (E) 100 mg/L; (F) infected control.

Effect of lactoferrin post treatment on AMV inhibition under greenhouse conditions:

The effect of lactoferrin posttreatment on the inhibition of alfalfa mosaic virus (AMV) under greenhouse conditions was investigated (**Table 2**). Lactoferrin at concentrations 100, 250, 500, 750 and1000 mg/L was applied, and observations were made after 21 days posttreatment. At the highest concentration of 1000 mg/L, when applied at 7 days post inoculation, significant inhibition was observed being 60% inhibition. Similar trends were observed in infected plants, where higher concentrations of lactoferrin (LF) correlated with increased inhibition of virus spread. Notably, at 1000 mg/L, 60% inhibition was achieved by day 7 posttreatment. In comparison to the inoculated control, where no inhibition was detected, the application of lactoferrin (LF) clearly suppressed AMV symptoms. Additionally, untreated uninoculated controls remained healthy throughout the observation period. These results suggest that lactoferrin (LF) has a dose-dependent effect on AMV inhibition, highlighting its potential as an antiviral agent against viral infections in greenhouse settings. The least significant difference (L.S.D.) values calculated at the 0.05 significance level provided insights into the reliability of the observed differences, indicating the precision of the experimental outcomes.

Table 2. Effect of lactoferrinposttreatment on AMV inhibition under greenhouse conditions**.**

Lactoferrinconc. (mg/ L)	Healthy	Infected	Inhibition (%)
100	1^{e}	19^{a}	5^{d}
250	5^{d}	15^{b}	25^{c}
500	8^{c}	12^{bc}	40^{b}
750	11^{b}	9^{cd}	55^{a}
1000	12^{b}	8^{d}	60^{a}
Inoculated control	0^{e}	20^{a}	0^{d}
Untreated uninoculated control	20^{a}	0^{e}	0^{d}
Treated uninoculated control	20^{a}	0^{e}	0^{d}
L.S.D at 0.05	2.09	3.43	5.76

Protein analysis

The impact of lactoferrin (LF) spray treatment administered five days prior to virus inoculation was investigated using markers as reference proteins. In contrast to those in the control treatment, the levels of newly synthesized proteins were significantly greater in the treated leaves (**Figure 4**). The application of lactoferrin (LF) at concentrations of 1000, 750, 500, and 250 mg/L resulted in detection of identical induced proteins with

molecular weights of 18, 27, and 30, which were detected in relatively similar quantities across all four concentrations. Notably, these proteins differed from those observed in both the inoculated control and healthy plants. Additionally, treatment with concentrations of 1000 and 750 mg/L induced the expression of proteins with molecular weights of 25 and 37 kDa. This suggests a distinct protein induction response to lactoferrin treatment, potentially indicative of specific plant defense mechanisms triggered by lactoferrin (LF) application prior to virus exposure.

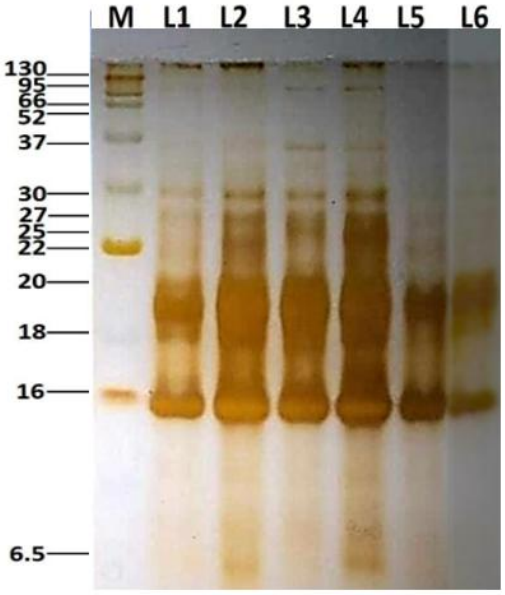

Fig. 4. SDS–PAGE analysis of total protein extracted from periwinkle leaves pretreated with varying concentrations of lactoferrin (LF). Lane M contains the molecular mass marker, while lanes L1 to L4 represent periwinkle leaves sprayed with lactoferrin at concentrations of 250 mg/L, 500 mg/L, 750 mg/L, and 1000 mg/L, respectively. Lane 5 contains unsprayed periwinkle leaves, and Lane 6 contains infected unsprayed periwinkle leaves.

Total phenol content and enzyme activity:

The impact of lactoferrin spray treatment on peroxidase (POX) and polyphenol oxidase (PPO) activity, as well as total phenol levels, in periwinkle plants inoculated with alfalfa mosaic virus (AMV) was assessed over a span of 7, 14, and 21 days (**Table 3**). Various concentrations of lactoferrin ranging from 100 to 1000 mg/L were applied. The results showed that as the lactoferrin concentration increased, there was a corresponding increase in POX and PPO activity, as well as in total phenol levels. At the highest concentration of 1000 mg/L, the least significant difference (L.S.D.) values calculated at the 0.05 significance level were used to evaluate the reliability of the observed differences, indicating the precision of the experimental outcomes.

Effects of various concentrations of lactoferrin on the levels of chlorophyll a,b and carotenoid in periwinkle leaves:

The impact of various concentrations of lactoferrin (LF) on the levels of photosynthetic pigments in periwinkle leaves over the course of 1, 3, and 5 days was investigated, as shown in **Table 4**. Across the concentration range of 100 to 1000 mg/L, lactoferrin (LF) had a dose-dependent effect on chlorophyll a, chlorophyll b, and carotenoid levels. At higher concentrations, there was a notable increase in the levels of these pigments. Specifically, at 1000 mg/L, the highest concentration tested, the chlorophyll a, chlorophyll b, and carotenoid levels peaked. Compared with those in the control groups, both inoculated and uninoculated periwinkle leaves treated with lactoferrin (LF) exhibited significantly greater pigment levels. The untreated uninoculated control (healthy) displayed higher pigments levels than the inoculated control, indicating the negative impact of the virus on pigment synthesis. Conversely, the pigment levels in the treated uninoculated control group were comparable to or greater than those in the untreated uninoculated control group, highlighting the beneficial effect of lactoferrin treatment. The least significant difference (L.S.D.) values calculated at the 0.05 significance level provided insights into the reliability of the observed differences, indicating the precision of the experimental outcomes.

Table 3. Impact of lactoferrin (LF) spray treatment on peroxidase (POX) and polyphenol oxidase (PPO) activity and total phenol levels in periwinkle plants inoculated with Alfalfa mosaic virus (AMV).

Lactoferrin (LF) Conc. (mg/ L)	POX (mg/g F.W)			PPO (mg/g F.W)			Total phenols (mg/100 g F.W)		
	7day	14day	21day	7day	14day	21day	7day	14day	21day
100	1.81^{c}	2.09^{c}	1.65^{b}	1.13^{d}	1.28^{c}	1.07^{d}	1.14^{d}	1.21^{d}	1.07^{c}
250	1.89^{c}	2.16^{c}	1.76^{b}	1.53^{c}	1.77^{b}	1.35^{c}	1.29^{c}	1.37^{cd}	1.19^{c}
500	2.13^{b}	2.28^{bc}	2.04^{a}	1.61^{c}	1.84^{b}	1.46^{c}	1.47^{b}	1.55^{bc}	1.39^{b}
750	2.29^{ab}	2.55^{ab}	2.18^{a}	2.41^{b}	2.60^{a}	2.24^{b}	1.56^{b}	1.76^{ab}	1.43^{b}
1000	2.39^{a}	2.77^{a}	2.23^{a}	2.61^{a}	2.79^{a}	2.51^{a}	1.81^{a}	1.95^{a}	1.79^{a}
Inoculated control	0.55^{d}	0.63^{d}	0.46^{c}	0.20^{e}	0.30^{d}	0.09^{e}	0.22^{e}	0.33^{e}	0.03^{e}
Untreated uninoculated control	0.25^{e}	0.26^{e}	0.27^{c}	0.17^{e}	0.16^{d}	0.18^{e}	0.21^{e}	0.20^{e}	0.23^{d}
Treated uninoculated control	1.71^{c}	2.02^{c}	1.54^{c}	1.03^{d}	1.13^{c}	1.01^{d}	1.05^{d}	1.14^{d}	1.02^{c}
L.S.D at 0.05	0.21	0.27	0.18	0.18	0.21	0.12	0.12	0.21	0.17

Table 4. Effect of different concentrations of lactoferrin (LF) on the levels of photosynthetic pigments in periwinkle leaves.

Lactoferrin Conc. (mg/ L)	Chlorophyll a (mg/g F.W)			Chlorophyll b (mg/g F.W)			Carotenoids (mg/g F.W)		
	Days								
	1	3	5	1	3	5	1	3	5
100	0.511^{a}	0.521^{b}	0.532^{cd}	0.335^{c}	0.343^{df}	0.352^{a}	2.332^{b}	2.349^{b}	3.378^{a}
250	0.529^{a}	0.535^{ab}	0.548^{bc}	0.346^{bc}	0.358^{cd}	0.370^{a}	2.355^{b}	2.367^{b}	2.379^{b}
500	0.543^{a}	0.550^{ab}	0.561^{ab}	0.362^{b}	0.373^{c}	0.382^{a}	3.303^{a}	3.323^{a}	3.338^{a}
750	0.551^{a}	0.562^{a}	0.576^{a}	0.385^{a}	0.391^{b}	0.409^{a}	3.313^{a}	3.339^{a}	3.348^{a}
1000	0.564^{a}	0.573^{a}	0.581^{a}	0.404^{a}	0.411^{a}	0.419^{a}	3.335^{a}	3.349^{a}	3.368^{a}
Inoculated control	0.355^{b}	0.354^{c}	0.352^{e}	0.213^{d}	0.209^{e}	0.211^{b}	1.723^{c}	1.720^{c}	1.721^{c}
Untreated uninoculated control	0.512^{a}	0.513^{b}	0.511^{d}	0.336^{c}	0.334^{f}	0.353^{a}	2.323^{b}	2.324^{b}	2.322^{b}
Treated uninoculated control	0.568^{a}	0.578^{a}	0.585^{a}	0.409^{a}	0.417^{a}	0.424^{a}	3.339^{a}	3.355^{a}	3.372^{a}
L.S.D at 0.05	0.103	0.036	0.027	0.021	0.015	0.100	0.355	0.322	0.312

Effects of various concentrations of lactoferrin on two active ingredients in periwinkle leaves.

The effects of different concentrations of lactoferrin (100, 250, 500, 750, and 1000 mg/L) on the levels of vinblastine and vincristine in periwinkle plants was evaluated over a span of 1, 3, and 5 days (**Table 5**). The results indicated a dose-dependent response, with higher concentrations of lactoferrin (LF) generally correlating with increased levels of both vinblastine and vincristine. At the highest concentration of 1000 mg/L, the levels of both compounds were significantly elevated across all three observation days compared to those at lower concentrations. In contrast, the inoculated control group showed markedly lower levels of vinblastine and vincristine throughout the assessment period. Similarly, untreated uninoculated controls displayed relatively stable levels of the two compounds. The least significant difference (L.S.D.) values calculated at the 0.05 significance level provided insights into the reliability of the observed differences, indicating the precision of the experimental outcomes.

Phylogenetic analysis

To validate the results of virus identification by RT–PCR, the PCR products were sequenced bidirectionally via the Sanger method and subsequently deposited in GenBank under accession number PP034583. The obtained sequence data were analyzed using MEGA 11 software and compared with 32 reference isolates. BLAST analysis indicated that the nucleotide identity of the AMV RAA23 isolates ranged from 92.8% to 99.3% with that of AMV isolates from various geographical regions (**Table 6**). Notably, sequence alignment of the CP revealed 99.3% identity between isolates from New Zealand (NZ34) and China (BJAp1). Phylogenetic analysis based on the neighbor-joining method revealed the partial sequence of the CP of the AMV isolate in this study. Furthermore, selected sequences of 32 previously characterized AMV isolates retrieved from the GenBank database clustered into four molecular groups, irrespective of their geographic origin or host plant (**Figure 5**). The first molecular group comprised isolates from Canada, Italy, Egypt, the USA, Turkey, New Zealand, China, Serbia, Bosnia and Herzegovina, Iran, the Netherlands, Australia, Croatia, and Korea, with identities ranging from 97.7% to 99.3% (**Table 6**). The Egyptian AMV isolate RAA23 fell within this group. The second group consisted of isolates primarily from Italy, the Netherlands, and Spain, exhibiting identities ranging from 94.6%

to 95.7%. In molecular group III, isolates from Egypt shared identities ranging from 92.8% to 93.4%, with 96.6% similarity to the AMV-Eggplant-EG isolate in molecular group IV. Phylogenetic analysis has provided valuable insights into virus evolution and genetic interactions.

Table 5. Effect of various concentrations of lactoferrin (LF) on vinblastine and vincristine in periwinkle leaves.

Lactoferrin	Vinblastine			Vincristine		
Conc.(mg/L)	1	3	5	1	3	5
100	0.221^{bc}	0.238^{bc}	0.255^{ab}	0.285^{a}	0.349^{ab}	0.363^{c}
250	0.301^{ab}	0.313^{ab}	0.325^{a}	0.306^{a}	0.359^{ab}	0.374^{bc}
500	0.319^{ab}	0.328^{ab}	0.339^{a}	0.329^{a}	0.367^{ab}	0.388^{ab}
750	0.345^{ab}	0.385^{a}	0.390^{a}	0.338^{a}	0.379^{a}	0.393^{ab}
1000	0.376^{a}	0.389^{a}	0.393^{a}	0.368^{a}	0.381^{a}	0.399^{a}
Inoculated control	0.155^{c}	0.158^{c}	0.156^{c}	0.149^{b}	0.152^{c}	0.147^{e}
Untreated uninoculated control	0.323^{ab}	0.321^{ab}	0.324^{a}	0.303^{a}	0.305^{b}	0.301^{d}
Treated uninoculated control	0.379^{a}	0.392^{a}	0.396^{a}	0.373^{a}	0.385^{a}	0.401^{a}
L.S.D at 0.05	0.121	0.109	0.130	0.109	0.058	0.018

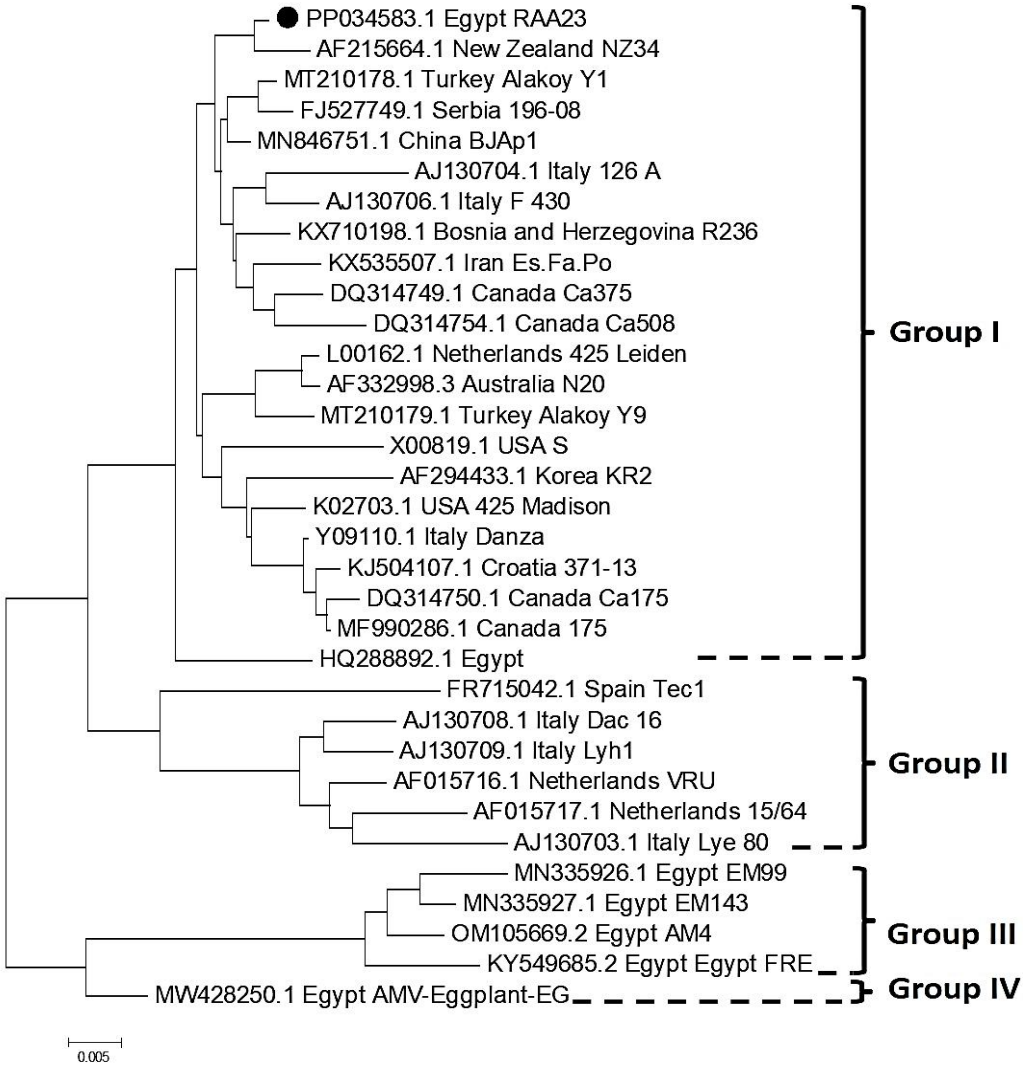

Fig. 5. Phylogenetic and molecular evolutionary analyses were conducted based on the partial nucleotide sequences of the CP gene of the AMV isolate and 32 reference AMV isolates using the neighbor-joining analysis method of MEGA version 11 (**Tamura *et al.* 2021**) via bootstrap analysis with 1000 replicates. The Egyptian RAA23 isolate is indicated by a black filled circle.

Table 6. The CP gene sequences of *Alfalfa mosaic virus* (AMV) isolates utilized in the phylogenetic analysis.

Accession number	Host	Isolate name	Country	Identity (%)
PP034583.1	Periwinkle	RAA23	Egypt	
AF215664.1	Pea	NZ34	New Zealand	99.3%
MN846751.1	Acyrthosiphonpisum	BJAp1	China	99.3%
MT210178	Medicago sativa	Alakoy Y1	Turkey	99.0%
KX710198.1	Capsicum annuum	R236	Bosnia and Herzegovina	98.9%
FJ527749.1	Nicotiana tabacum	196-08	Serbia	98.9%
KX535507.1	Potato	Es.Fa.Po	Iran	98.5%
AJ130706.1	Nicotiana glutinosa	F 430	ITALY	98.5%
DQ314749.1	Potato	Ca375	Canada	98.5%
Y09110.1	Lycopersicon esculentum	Danza	ITALY	98.4%
K02703.1	Nicotiana tabacum	425 Madison	USA	98.4%
MT210179	Medicago sativa	Alakoy Y9	Turkey	98.2%
L00162.1	Nicotiana tabacum	425 Leiden	Netherlands	98.0%
MF990286.1	Solanum tuberosum	175	Canada	98.0%
KJ504107.1	Lavandula x intermedia	371-13	Croatia	98.0%
AF332998.3	N. clevelandii	N20	Australia	98.0%
DQ314754.1	Potato	Ca508	Canada	97.9%
AF294433.1	Solanum tuberosum	KR2	Korea	97.7%
X00819.1	Nicotiana tabacum	S	USA	97.7%
DQ314750.1	Potato	Ca175	Canada	97.7%
AJ130704.1	Nicotiana glutinosa	126 A	ITALY	97.7%
HQ288892.1	S. tuberosum	Egyptian	Egypt	97.7%
MW428250.1	Eggplant	AMV-Eggplant-EG	Egypt	96.6%
AF015716.1	Nicotiana tabacum	VRU	Netherlands	95.7%
AJ130708.1	Nicotiana glutinosa	Dac 16	ITALY	95.4%
AJ130709.1	Nicotiana glutinosa	Lyh 1	ITALY	95.4%
AF015717.1	Garden lupin	15/64	Netherlands	94.9%
AJ130703.1	Nicotiana glutinosa	Lye 80	ITALY	94.6%
FR715042.1	Tecomaria capensis	Tec1	Spain	94.6%
OM105669.2	Trifolium repens	AM4	Egypt	93.4%
MN335927.1	Solanum tuberosum	EM143	Egypt	93.3%
MN335926.1	Solanum tuberosum	EM99	Egypt	92.9%
KY549685.2	Potato	FRE	Egypt	92.8%

DISCUSSION

The viral strain examined in this study was isolated from periwinkle plants obtained from Giza Governorate, Egypt. Characteristic symptoms of *Alfalfa mosaic virus* (AMV) infection, such as bright yellow blotches, vein banding, net yellow patterns, and chlorosis, were observed on the leaves, consistent with previous reports (Sofy *et al*., 2021). The virus isolate demonstrated efficient mechanical transmission from infected plants to healthy greenhouse-grown periwinkle seedlings, with systemic symptoms manifesting 15-21 days postinoculation, corroborating findings from similar studies on Kura clover (*Trifolium amgibuum*) infected with AMV (Pineyro *et al*., 2002). Notably, AMV is known to infect a broad spectrum of hosts, spanning more than 600 plant species globally. In Egypt, AMV has been shown to infect various hosts, including *Catharanthus roseus*, commonly known as periwinkle. Given the propensity for AMV to infect periwinkle plants, there is a potential risk for the virus to serve as a reservoir, facilitating its transmission to other ornamental and economically significant crops.

The primary discovery of this study revolves around lactoferrin (LF), a cost-effective, nontoxic, and environmentally sustainable compound. The initial mechanism of action of lactoferrin against viruses involves its binding to cell receptors, subsequently hindering the entry of DNA- or RNA-based viruses into cells (Van der Strate *et al*., 2001). This antiviral activity may be attributed to the increased hydrophobicity of lactoferrin resulting from esterification and its high isoelectric point. Preliminary test results suggest a proportional relationship between the concentration of lactoferrin (LF) and its antiviral efficacy against AMV. Previous studies have described lactoferrin (LF) as an antiviral agent, and its enhanced antiviral properties through esterification are attributed to a heightened net positive charge and hydrophobicity (Sojar *et al*., 1998; Osman *et al*., 2014; Abdel-Shafi *et al*., 2016).

The aim of this study was to mitigate the damage inflicted on infected periwinkle plants by applying varying concentrations of lactoferrin (LF) by spraying under greenhouse conditions. Notably, the concentration of 1000 mg L^{-1} exhibited the highest effectiveness compared to the other concentrations. It is evident that preventive spray treatment, administered 3 or 5 days pre inoculation, enhances the inhibitory effects of lactoferrin concentrations. These findings align with those reported by Taha *et al*., (2015) regarding the efficacy of lactoferrin (LF) against *Potato virus X* in potatoes. Previous research has demonstrated that spraying plants with 0.1% lactoferrin (LF) provides protection against potato virus X in potatoes and against *Tomato yellow leaf curl virus* in tomatoes (Abdelbacki *et al*., 2010). Additionally, Wang *et al*., (2013) reported that incubating *Tobacco mosaic virus* inoculum with 0.1% lactoferrin (LF) for 30 minutes resulted in an 81% reduction in TMV infection in tobacco plants. Furthermore, Chanda *et al*., (2021) reported that treatments with 0.5% lactoferrin (LF) effectively shield watermelon and tomato plants from both *Cucumber green mottle mosaic virus* and *Tomato brown rugosa fruit virus*.

In the present study, periwinkle plants treated with lactoferrin (LF) and subsequently infected with AMV exhibited elevated levels of photosynthetic pigments. The application of lactoferrin (LF), whether administered 1, 3, or 5 days prior to virus inoculation, resulted in increased quantities of chlorophyll a, chlorophyll b, and carotenoids, whereas infected control plants displayed reduced pigment concentrations. As noted by Tecsi *et al*., (1996), virus infection often leads to characteristic symptoms such as vein banding, chlorosis, or overall yellowing of leaves, which may contribute to alterations in chlorophyll and carotenoid levels. Given their vital role in plant growth and development, a decrease in chlorophyll content is significant because chlorophyll serves as a chlorophyll synthesis inhibitor (Sutic and Sinclair, 1990). The enhanced content of chlorophyll and carotenoids following lactoferrin treatment may be attributed to the antioxidant properties of these compounds, which potentially shield chlorophyll molecules and prevent the degradation of carotenoids (Ashraf *et al*., 2023).

The application of lactoferrin (LF) resulted in heightened activity of defense enzymes such as peroxidase and polyphenol oxidase, as well as increased phenol levels. Phenolic compounds are recognized as pivotal components of a plant's defense system against diverse external pathogens. The induction of resistance is correlated with the accumulation of salicylic acid, which promotes the expression of pathogenesis-related proteins, activates the phenylpropanoid pathway, and promotes the synthesis of phenolic substances, which are crucial for combating viruses (Din Umar *et al.,* 2016). Phenolic metabolism and cell wall lignification play integral roles in various ecological and cellular processes, potentially enhancing plant resistance to detrimental agents. Phenolic acids contribute to phytoalexin accumulation, lignin synthesis, and the establishment of structural barriers, which are all pivotal for pathogen resistance (Sudhakar *et al*., 2007).

Compared with untreated plants, lactoferrin-treated periwinkle plants exhibited elevated peroxidase and polyphenol oxidase activity. This increase could stem from heightened expression of specific defense genes or possibly from an initial signal triggered by infected leaves activating enzymes that fortify plant cell walls and aid in protein extension, forming a firmer matrix as part of the activated defense response (Jabs *et al*., 1996). Peroxidase facilitates the final step in lignin and oxidative phenol formation, playing a significant role in phenolic substance oxidation (Aldesuquy *et al*., 2015). Polyphenol oxidase, which converts phenolic compounds into quinones and hinders virus growth by deactivating RNA, can be stimulated through the octadecanoid defense signaling pathway (Constable *et al*., 1995).

Treating periwinkle plants with lactoferrin (LF) resulted in an increase in the vinblastine and vincristine contents, whereas infection with AMV led to a reduction in these substances. Various studies have shown that elicitors effectively enhance the production of secondary metabolites in medicinal plants (Ionkova, 2007; Simic *et al*., 2015; Yu *et al*., 2016). Among the strategies for augmenting secondary metabolite synthesis, the use of elicitors is crucial (Esmaelizade and Sharifi, 2013). Elicitors, whether they are biological or chemical agents, can induce defensive reactions and physiological changes, ultimately fostering the biosynthesis and accumulation of secondary metabolites (Ramezani *et al*., 2018).

Cathranthus mosaic virus (CatMV) has been shown to hinder the production of vinblastine and vincristine, which are vital antineoplastic alkaloids found in *Catharanthus roseus*. However, the application of rhizobacteria to promote plant growth resulted in increased alkaloid content in periwinkle plants compared to infected control plants. Viral and biotic stimuli are known to stimulate the immune system of the periwinkle, thereby enhancing the production and accumulation of vinblastine and vincristine.

Certain pathogenesis-related proteins (PRs) have demonstrated antipathogenic properties (Van Loon *et al*., 1994). The accumulation of new proteins is crucial for the buildup of pathogenesis-related proteins, which play a pivotal role in establishing robust systemic resistance against viruses (Devi *et al*., 2004). Consistent with this hypothesis, Harish *et al*., (2004) reported increased resistance to *Banana bunchy top virus* in plants treated with endophytic bacteria, accompanied by increased activity of defense-related proteins such as chitinase. The

presence of biotic agents often leads to the elevation of numerous PR proteins, including chitinase and peroxidase isozymes (Neetu *et al.,* 2008).

Elicitors have been linked to an increase in pathogenesis-related proteins with molecular weights of 24 and 33 kDa, which are associated with enhanced plant resistance against tobacco necrosis virus (Shoman et al., 2003). A 30 kDa band, potentially belonging to the chitinase family, was observed, aligning with assumptions made by Santos *et al*., (2004). This suggests that elevated concentrations of the pathogenesis-related protein chitinase could contribute to the absence of disease symptoms in periwinkle plants. SDS–PAGE analysis revealed new protein patterns following lactoferrin treatment of periwinkle leaves, with estimated molecular weights of 18, 25, 27, 30, and 37 kDa. It is plausible that intercellular proteins that accumulate in resistant plants serve as the first line of defense against challenging diseases. These proteins are associated with plant defense due to their antiviral properties (Van Loon *et al*., 1994).

The study identified nucleotide sequences of the AMV isolate RAA23, revealing sequence identities ranging from 92.8% to 99.3%. Sequence alignment of the coat protein (CP) indicated a close relatedness to the NZ34 and BJAp1 isolates from New Zealand and China, respectively. Phylogenetic analysis of the CP gene depicted the division of the AMV isolate RAA23 and 32 other isolates into four distinct molecular groups. This analysis aligns with previous studies by Xu and Nie (2006), Stanković *et al.*, (2011), and Topkaya (2022), who also categorized AMV isolates into four distinct molecular groups. However, Parrella *et al*., (2010) categorized AMV isolates into two groups (I and II), with the second group further divided into IIA and IIB by Parrella *et al*., (2011). The observed differences may be attributed to geographical variations or differences in the amino acid sequences of the CPs, which are potentially linked to the structural characteristics of the viral particles. Notably, Xu and Nie (2006) reported the presence of AMV isolates in various groups regardless of their regional distribution.

CONCLUSION

Alfalfa mosaic virus (AMV) was isolated from naturally infected periwinkle plants in Giza Governorate, symptoms observed included yellow blotches, vein banding, and chlorosis. The virus was purified biologically and virus identification involved various methods, including symptomatology, transmissibility, DAS ELISA, electron microscopy, and molecular detection. Lactoferrin (LF) was used at different concentrations to mitigate AMV infection, the highest LF concentration resulted in a 90% reduction in viral infection moreover led to significant increases in phenol levels and enzyme activity in addition to induced production of new proteins and the maximum levels of vinblastine and vincristine were detected at the highest LF concentration and increased the concentrations of photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoids. This research provides valuable insights into AMV behavior and sheds light on potential strategies for managing AMV infections and highlights the role of LF in modulating plant responses.

Funding: Agricultural Research Center, Giza, Egypt.
Conflict of Interest: The authors declare no conflict of interest

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