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Evaluation of *Clitoria ternatea* flower extracts as a novel method to manage *Bean yellow mosaic virus* (BYMV) infecting Faba Bean

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ABSTRACT

Bean yellow mosaic virus (BYMV) is one of the most destructive viral diseases affecting leguminous crops, causing severe yield losses. This study aimed to evaluate the potential activity of *Clitoria ternatea* flower extracts both alcohol and aqueous at different concentrations as a novel method to manage BYMV infection in faba bean plants. The incidence of BYMV was recorded in four governorates (Fayoum, Beheira, Giza, and Bani Suef) during the 2022 growing season to determine the epidemiology of the virus, with infection rates ranging from 14.4 to 46.4%. BYMV was isolated and identified based on the symptoms, host range and molecular techniques. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect BYMV, 54 out of 95 samples gave positive results, amplicons were approximately 907bp using specific BYMV primer and 335bp with degenerate primer. Sequence analysis of the Egyptian isolate (Accession No. PP481923) showed identity ranging from 90.8% to 98.3% compared with ten BYMV sequences in GenBank. Various treatments with *C. ternatea* flower extracts were applied. Results demonstrate that the application of *C. ternatea* extracts, particularly the alcohol extract in protective treatment at a concentration of 3% effectively eliminated virus-induced symptoms, improved growth parameters, and enhanced photosynthetic pigments in infected faba bean plants compared to the untreated infected control. These results were confirmed by real-time RT-PCR. The flower extracts haven't been previously used to manage plant viruses, while they have been used to resist human viruses *i.e. Coronaviruses*. Using *C. ternatea* flower extracts is a promising eco-friendly and alternative control method against BYMV.

Keywords: *Bean yellow mosaic virus* (BYMV), RT-PCR, sequencing, *Clitoria ternatea*, photosynthetic pigments and Real-time RT-PCR.

INTRODUCTION

Faba bean (*Vicia faba* L.) is an important crop in Egypt, and it is mainly grown in the Nile Valley and Delta regions. The faba bean total cultivated area in Egypt was approximately 90.770 and 6730 feddans with a total production of 130.093 and 46.406 tons for dry and green faba bean respectively in season 2022 according to (Agricultural Statistics Bulletin, Ministry of agriculture-Economic affair sector). Faba bean used for human consumption due to its nutritional values including proteins, minerals and vitamins, also used as forage for livestock (Liu *et al*., 2022).

Bean yellow mosaic virus (BYMV) is one of the most important plant viruses that limit faba bean production worldwide, causing significant yield losses and reduced seed quality (Omar 2021). BYMV is a member of the genus potyvirus, has a positive-sense single-stranded RNA genome of approximately 10 kb and flexuous rod-shaped filaments (Wylie *et al*., 2017; Yang *et al*., 2021; Mrkvová *et al.,* 2024). It associated with various symptoms on faba bean such as mosaic, mottle, mild mosaic, flexing, green vein banding, leaf rolling, (Zeid 2016; Moury and Desbiez 2020; Younes *et al*., 2021). BYMV consider the most widespread viruses that attack faba bean crops in Egypt. Depending on the time of BYMV infection the percentage of yield loss vary from 15 to 45% (Kaiser 1973 and Makkouk *et al*., 1988). BYMV induced 33% reduction in the number of pods per plant and a 41% reduction in seed yield (Hampton 1975). In Egypt 55.5% reduction in the number of pods per plant was reported (Allam *et al*., 1979). Reverse transcription polymerase chain reaction (RT-PCR) is a sensitive method successfully employed to detect *Bean yellow mosaic virus* in the infected plants (Uga *et al*., 2004; Duraisamy *et al*., 2011). Virus phylogenetic analysis is essential for tracking a virus's evolutionary path through time and for comprehending the host ranges of different strains in order to prevent new epidemics and control viruses. Thus far, most BYMV phylogenetic investigations have relied on the partial sequencing of

a genomic area or the sequence of a single gene to infer the relationships between the strains (Baradar *et al*., 2021; Kaur *et al*., 2018; Al-Khalaf *et al*., 2008).

Various methods have been used to control the virus, including chemical treatments. However, these methods have restrictions such as environmental toxicity and insecticides resistant. Therefore, there is a need to explore alternative methods for controlling BYMV in faba bean. Plant viral control through increasing plant systemic immunity is an effective and eco-friendly approach (Abdelkhalek *et al*., 2022). One potential approach is the use of natural biocontrol agents, such as plant extracts, which are safe, effective, and environmentally friendly (Rana and Singh 2021).

Clitoria ternatea L. is known as Butterfly Pea, Korrdofan Pea (Sudan), Cunha (Brazil), or Pokindong (Philippines). This plant is a member of Leguminosae (Fabaceae) family, commonly grown as an ornamental plant or forage legumes (Kosai *et al*., 2015). *C. ternatea* is native to Southeast Asia but is now cultivated in tropical and subtropical environments worldwide (Mukherjee *et al*., 2008; Kosai *et al*., 2015; Oguis *et al*., 2019 and Muchugi, 2023). The plant roots, stems, leaves, flowers, and seeds are commonly used in traditional medicine, diets and can also used as a natural dye, food colorant, and in the cosmetic industry (Havananda and Luengwilai 2019). An analysis of the nutritional composition of *Clitoria ternatea* flowers revealed that they contain approximately 0.32% protein, 2.1% fiber, 2.2% carbohydrates, and 2.5% fat. The moisture content of the flowers was measured to be 92.4%. Additionally, the flowers were found to possess significant amounts of calcium (3.09 mg/g), magnesium (2.23 mg/g), potassium (1.25 mg/g), zinc (0.59 mg/g), sodium (0.14 mg/g), and iron (0.14 mg/g) (Neda *et al*., 2013). The bioactive compounds of *C. ternatea* plant are common in the form of polyphenols, triterpenoids, flavonoids (anthocyanin, delphinidin, ternatin, kaempferol, catechin, epicatechin, quercetin, preternatin, myrisetin and syringetin), glycosides, tannins, steroids, and protein (Kazuma *et al*., 2003; Neda *et al*., 2013; Nair *et al.,* 2015; Azima *et al*., 2017; Minelko *et al*., 2020 and Kumar and Raj 2024).

Several studies identified and isolated various bioactive compounds from *C. ternatea* flower extract. These phytochemicals exhibit a wide range of pharmacological properties, including antioxidant, anti-inflammatory, antidiabetic, antimicrobial, antipyretic, analgesic and neuroprotective effects (Gupta *et al*., 2010; Mohandas and Anu 2013; Al-Snafi 2016; Yanti *et al*., 2020 and Ratha *et al*., 2023). According to studies on antimicrobial activity of *C. ternatea* extracts, it has antibacterial effects on numbers of bacteria species like *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Aeromonas formicans*, *Aeromonas hydrophila Streptococcus agalactiae*, *Salmonella typhi*, *Streptococcus mutans*, *Bacillus cereus*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Morganella morgani* (Ponnusamy *et al*., 2010; Setiawan *et al*., 2021; Jeyaraj *et al*., 2022, and Sathyanarayana *et al*., 2024) and antifungal effect on some fungi (Umamaheswari *et al*., 2010, and Yolin Angel *et al*., 2024). In addition, recent studies have shown that the plant possesses antiviral activity against coronaviruses (Nugraha *et al*., 2021, Fazadini and Yzzuddin 2022, Torres *et al.,* 2022 and Chun *et al.,* 2023). This study aims to evaluate the potential activity of C. *ternatea* flower extracts as a novel biocontrol agent to manage BYMV in faba bean based on their effects on disease incidence and severity, virus accumulation, and growth indices in faba bean plants.

MATERIAL AND METHODS

Incidence of BYMV and samples collection:

A Field survey was done in four governorates, Fayoum, Beheira, Giza, and Bani Suef during 2022 growing season to study the distribution and epidemiology of BYMV in different Faba bean growing fields (new reclaimed and old fields). In each governorate, four districts were surveyed and Faba bean fields were randomly selected for visual assessment and samples collection. In each selected faba bean field, five points were randomly selected and 20 plants were visually assessed at each point then the incidence was computed using the following formula (El-Gamal *et al*., 2022):

$$\text{Disease incidence (DI \%)} = \frac{\text{Number of infected plants}}{\text{Total number of assessed plants}} \times 100$$

Moreover, the incidence of the virus was also estimated using RT-PCR. Plant samples displaying symptoms such as mosaic, chlorosis, leaf rolling, green vein banding, stunting, and leaf distortion were collected through the vegetative stage (Fig. 1), and all collected samples were tested using RT-PCR.

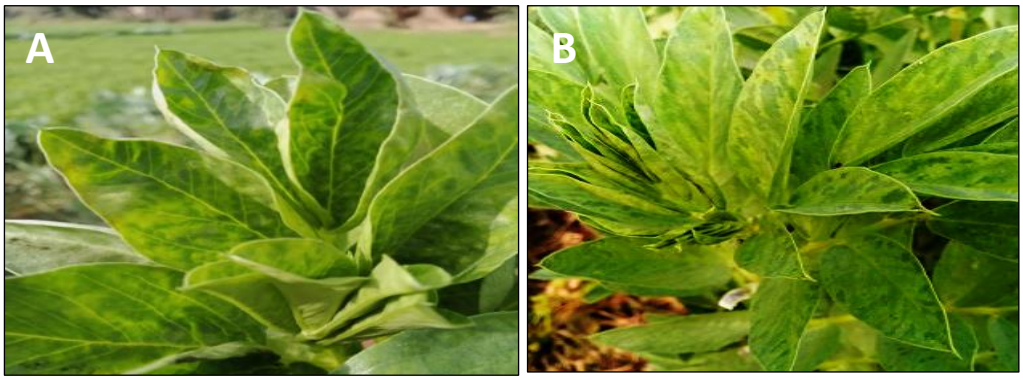

Fig. 1: Naturally infected faba bean plants with Bean Yellow Mosaic virus showing, (A): systemic mosaic and leaf rolling; (B): green vein banding, leaf distortion and stunting.

Virus isolation and Host range study:

Samples that gave positive results with RT-PCR reaction were used as a source for virus isolation. The single local lesion technique was used to produce a pure isolate of BYMV as described by Kuhn 1964. *Chenopodium amaranticolor* L. plants were mechanically inoculated with sap extracted from faba bean plantssystemically infected with the BYMV. The sap was prepared in phosphate buffer (1:5 w/v, 0.1 M, 0.1 mL, pH 7.2). Plants belonging to family *Leguminasea* (5 species), *Chenopodiaceae* (2 species), *Solanaceae* (5 species) and *Cucurbitaceae* (4 species) were used to study the host range. Inoculated plants were maintained in an insect-proof greenhouse until symptoms developed. Results were confirmed by RT-PCR detection.

Detection by reverse transcription-polymerase chain reaction (RT-PCR):

Total RNA extraction:

Total RNA was extracted from tested samples using Simply P Extraction Kit (DNA-free) Bioflux, China, according to the manufacturer and total RNA extraction was kept at - 20°C until detection.

RT-PCR detection:

RT- PCR was carried out using Verso 1-Step RT-PCR Kit ReadyMix (ThermoPrime Taq). All components required for both reactions were added during setup. The Oligonucleotide Specific and/or degenerate primers sequence used to detect BYMV by RT-PCR was designed based on the sequence of the coat protein (CP) gene as shown in Table 1 (Langeveld *et al*., 1991 and Mohamad *et al*., 2008). Amplification was performed in an automated thermal cycler (Proflex Applied Biosystems gene) programmed for the thermo-cycling conditions for specific primer BYMV-CPU, BYMV-CPD 50ºC for 15 min for cDNA synthesis, 5 min at 94ºC for reverse transcriptase inactivation and initial denaturation, followed by 35 cycles of 60 s at 94ºC, 1 min at 50ºC and 2 min at 72ºC, and final extension for 5 min at 72ºC, and for degenerate primer U335, D335: 50ºC for 15 min for cDNA synthesis, 5 min at 94ºC for reverse transcriptase inactivation and initial denaturation, followed by 45 cycles of 30 s at 94ºC, 5 min at 60ºC and 1 min at 72ºC, and final extension for 5 min at 72ºC, RT-PCR amplified DNA fragments were separated at 100 v/45 min in 1% agarose gel electrophoresis in 0.5x TAE buffer after stained by AZView stain Biomatik Canada, and visualized with UV illumination.

Real-time RT-PCR with SYBR® green detection:

qRT-PCR was performed on QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, USA) using specific primers as described by Duraisamy *et al*., 2011 (Table 1) to evaluate the effect of *Clitoria ternatea* flowers extracts treatments on BYMV titer. The qRT-PCR mix was made using Verso 1-Step qRT-PCR SYBR ROX Mix (Thermo Fisher Scientific, USA) in 25 µl containing 3 µl of RNA template, 12.5 µl Verso 2X 1-Step RT-qPCR SYBR mix, 1.5 µl of each forward and reverse primer, 1.25 µl RT Enhancer and 0.25 µl Verso Enzyme mix and 5 µl distilled water. The cycling conditions were as follows: 15min of reverse transcription at 50°C and 5min of polymerase activation at 95°C followed by 40 PCR cycles of 30 s at 95°C for denaturation and 30 s at 54°C for annealing and 72°C for 30 s, and finally an extension at 72°C for 10 min. Each sample was tested and repeated independently three times. Amplification specificity was checked using a heat dissociation protocol (melting curves in the range of 79.5 – 80.95°C).

Gel cleaning and sequencing:

After gel electrophoresis, the gel was excised and purified using the FavorPrepTM Gel Purification Mini Kit. Purified fragments of degenerate primer were sent for sequencing at Macrogen, Korea. Sequence alignment and similarity were calculated using DNAMAN software program version 7.0 (Lynnon BioSoft, Canada).

Table 1. Primers used for reverse transcription-polymerase chain reaction (RT-PCR) to detect Bean Yellow Mosaic virus (BYMV).

Primer	Sequence (5`-3`)	Product size (bp)	Ref.
U335	GAATTCATGRTNTGGTGYATHGANAAYGG	335	Langeveld *et al.,* 1991
D335	GAGCTCGCNGYYTTCATYTGNRHDWKNGC		
BYMV-CPU	GTCGATTTCAATCCGAACAAG	907	Mohamad *et al.,* 2008
BYMV-CPD	GGAGGTGAAACCTCACTAATAC		
NIF	GAGCGCATCGTTTCAATTCT	100	Duraisamy *et al.,* 2011
NIR	AGCATGGGGCTATCCAACT		

***Clitoria ternatea* flower extracts preparation:**

Drying process:

Petals of the vivid blue flower were obtained from the Fayoum Experimental Research station. The flowers were washed with distilled water and oven-dried overnight at 40 ± 2°C until a constant weight was achieved as described by Baskaran *et al*. (2019). The dried flowers were ground into a fine powder using a blender and stored in a dry clean container until extraction.

Extraction process:

Two different methods, alcoholic and aqueous extractions, were utilized to carry out the flower sample extraction, following the procedures described by Mohamad *et al*., (2018) and Baskaran *et al*. (2019), respectively with some modifications.

In the alcoholic approach, the dried flowers (1 g) were soaked in 25 mL of 50% ethanol overnight at room temperature, under dark conditions on a shaker incubator. The mixtures were then filtrated. Subsequently, alcohol was removed from the filtrated solution using a rotary evaporator by evaporation under reduced pressure at a relatively low temperature (25°C). In contrast, the aqueous approach involved adding sterilized distilled water as solvent into the dried flowers (1:25) overnight, after filtration the liquid was dried in the oven at 60°C overnight to remove water content.

Experimental greenhouse design and treatments application :

This experiment was conducted in an insect-proof greenhouse during the 2023 growing season, with controlled temperature conditions of 28°C during the day and 16°C at night. Healthy seeds of the Faba bean cultivar Giza 40 were obtained from the Egyptian Agriculture Ministry. The seeds were sown after being surface-sterilized in 40 cm diameter pots (3 seedlings/pot), filled with sandy-loam soil sterilized using an autoclave at 121^{o}C prior potting. Two experiments were designed in a complete randomized block design (CRBD) with three replicates for each treatment. The first experiment was to apply the treatments of *Clitoria ternatea* flowers alcoholic extract, while the second was to apply aqueous extract treatments, where three concentrations of 1%, 2% and 3% used for each extract. In each experiment the plants were divided into five groups after 10 days of growth. The groups were divided as follows: The first treatment group, Faba bean plants were without a viral inoculation and foliar sprayed with a sterile nutrient (Healthy control; HC). The second treatment group, Faba bean plants were mechanically inoculated with BYMV and then foliar sprayed with a sterile nutrient (Infected control; IC). The third group, the plants were foliar sprayed with extracts only (Negative control; NC). The fourth group includes faba bean plants that were foliar sprayed with extracts (alcoholic or aqueous) and then inoculated after 24 hours with BYMV (protective treatment). The fifth group was the BYMV-inoculated plants treated by the foliar spraying of *Clitoria ternatea* flowers extract at the beginning of BYMV-related symptoms appearance on faba bean (curative treatment).

Estimating disease incidence and severity:

The percentage of disease incidence was recorded for all treatments based on the visual symptoms observed on inoculated plants, using the formula as mentioned above. Disease severity was estimated using the disease severity index (DSI) as described in Figure 2. The disease severity values caused by BYMV were calculated using the following equation as described by Yang *et al.* (1997).

$$DSI\% = \frac{\Sigma(\text{Disease grade x No of plant in each grade})}{\text{Total no of tested plants x highest disease grade}} x100$$

Fig. 2: Disease severity index (DSI): index from 0 to 4 as the following scale: 0= no symptoms; 1= mild mosaic; 2= moderate mosaic; 3= severe mosaic, 4= pronounced leaf deformation and mosaic.

Growth parameters:
Three weeks after treatments, faba bean plants were carefully uprooted from the pots and vegetative growth parameters included, shoots height (cm), number of leaves per plant, branches per plant, number of flowers per plant, and number of Pods per plant. The shoots were weighed to estimate shoots fresh weight (g), after that, they were oven-dried at 70±2°C until a constant weight was reached (dry weight).

Determination of photosynthetic pigments:
The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were estimated by spectrophotometric method, and 0.5 g of leaves tissue (fresh weight) was homogenized in 85% acetone (5 mL) for 5 min. The homogenate was centrifuged and the supernatant was made up to a volume of 25 mL with 85% acetone. The extraction was measured against a blank of pure 85% aqueous acetone at three wavelengths (452.5, 644 and 663 nm) using a spectrophotometer nano-drop one^{C} (Applied Biosystems). The concentration of the pigment fraction (chlorophyll a, chlorophyll b and carotenoids) as µg/mL was determined using the following equations (Metzner *et al*., 1965 and Ibrahim *et al*., 2007)
Chlorophyll a (µg/mL) = 10.3 E663 – 0.918 E644
Chlorophyll b (µg/mL) = 19.7 E644 – 3.870 E663
Carotenoids (µg/mL) = 4.2 E452.5 – 0.0264 Chl a + 0.426 Chl b
Where, E is the extinction coefficient at the given wavelength. The concentration of pigment was calculated as mg/g fresh weight of plant leaves.

Statistical Analysis:
All the obtained data of disease incidence and severity, growth traits, and photosynthetic efficiency were analyzed through analysis of variance (ANOVA) using Microsoft Excel® 2016 and Genstat (11th edition, VSN International Ltd., Oxford, UK). Duncan as a post-hock test ($p \leq 0.05$* or $p \leq 0.01$**) was used as the mean separation test, and all the data are presented as means ± their standard errors (SEs).

RESULTS

Incidence and distribution of BYMV:
Symptomatic faba bean plants exhibited severe mosaic, stunting and upward leaf rolling (Fig. 3A) in the districts of Bani Suef Governorate, with incidence rates ranging from 14.4 to 46.4% based on visual inspection. A high incidence of BYMV (60%) was observed in a few fields. BYMV was also observed with incidence of 17.4 to 35.8% in Fayoum Governorate, symptomatic plants showed symptoms of chlorosis and vein banding (Fig. 3B). In addition to symptoms of mosaic, leaf deformation, and upward leaf rolling were recorded (Fig. 3C) in Giza Governorate with incidence from 18 to 29.3%. However, the lowest incidence was recorded in Beheira Governorate ranged from 4 to 18.6%. Leaf samples were collected from four governorates, RT-PCR detection gave positive results for 54 out of 95 samples (Table 2). The highest percentage of infection depending upon RT-PCR results was recorded in Bani Suef Governorate (65.6%) while the lowest infection rate was found in Beheira Governorate (40%).

Table 2: Incidence of *Bean yellow mosaic virus* (BYMV) in four Egyptian governorates during 2022 growing season.

Governorate	Districts/ Village	No. of fields surveyed	Incidence % based on visual inspection	Symptoms	No. of positive samples by RT-PCR/ Total	% of infection based on RT-PCR
Fayoum	Itsa	15	20%	CL, VB	12/20	60%
	Ibsheway	10	17.4%			
	Sinnuris	15	31.2			
	Tamiya	12	35.8			
Bani Seuf	Biba	8	38.5	LR, SM, LD,S	23/35	65.6
	El Fashn	12	46.4			
	Nasser	10	30.3			
	El Wasta	15	14.4			
Giza	El Badrashein	15	20.8	CL, LD	11/20	55
	El Ayyat	10	29.3			
	El Marazek	12	18			
	Kafr Hakeem	15	19.4			
Beheira	Wadi El Natrun	12	18.6	M, LD	8/20	40
	El Nubaria	10	11.2			
	Damanhour	10	4			
	Kom Hamada	8	15			

CL: chlorosis, VB: vein banding, SM: severe mosaic, LR: leaf rolling, M: mosaic, S: stunting, LD: leaf deformation.

Virus isolation and host range study:

BYMV was successfully isolated and purified from naturally infected plants. Host range studies showed that 8 out of 16 species were infected and exhibiting different symptoms, as presented in Table (3). *Lupines termis* showed vein greening, leaf narrowing and plant stunting. Symptoms in *Medicago sativa* L. were vein clearing, lethal systemic wilt and severe systemic mosaic. Leaf deformation on *Vicia faba,* chlorosis on *Phaseolus vulgaris* L.*,* mosaic on *Vigna unguiculata* and vein banding on *Pisum sativum* while Family *Chenopodiaceae* such as *Chenopodium amaranticolor* and *Chenopodium quinoa* showed symptoms of chlorotic local lesions on leaves (Fig. 4).

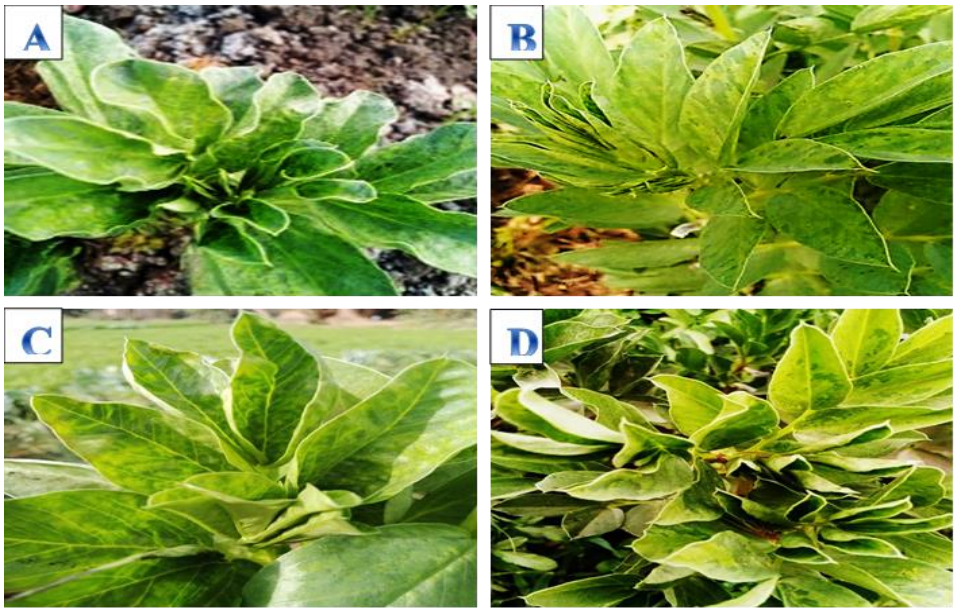

Fig. 3: *Bean yellow mosaic virus*-like symptoms observed in some fields during survey, **A:** severe mosaic, upward leaf rolling and stunting; **B**: chlorosis and vein banding; **C:** upward leaf rolling, mosaic; **D:** chlorosis and leaf malformation.

Table 3. Plant responses and susceptibility to mechanical inoculation with BYMV under greenhouse conditions.

Host plant tested			Symptoms induced	RT-PCR
Family	**Scientific name**	**English name**		
Leguminosae	*lupines termis*	Lupine	VG, LN, S	+
	Medicago sativa L.	Alfalfa	VC, LSW	+
	Pisum sativum L.	Garden pea	M	+
	Vicia fabae L.	Broad bean	M, LD	+
	Phaseolus vulgaris L.	Common bean	CL	+
	Vigna unguiculata	Black-eye pea	M	+
Chenopodiaceae	*Chenopodium amaranticolor*	Lambsuarters	CLL	+
	Chenopodium quinoa	Goosefoot	CLL	+
Cucurbitaceae	*Cucumis sativus*	Cucumber	NS	-
	Cucurbita pepo	Squash	NS	-
Solanaceae	*Solanum tuberosum*	Potato	NS	-
	Nicotiana glutinosa L.	Tobacco plant	NS	-
	Solanum lycopersicum	Tomato	NS	-
	Capsicum annuum L.	Pepper	NS	-
	Solanum nigrum	Black nightshade	NS	-
	Datura stramonium	Devil's trumpet	NS	-

VG: vein greening, LN: leaf narrowing, S: stunting, VC: vein clearing, LSW: lethal systemic wilt, M: mosaic, LD: leaf deformation, CL: chlorosis, CLL: chlorotic local lesion, NS: no symptoms, +: positive reaction and -: negative reaction.

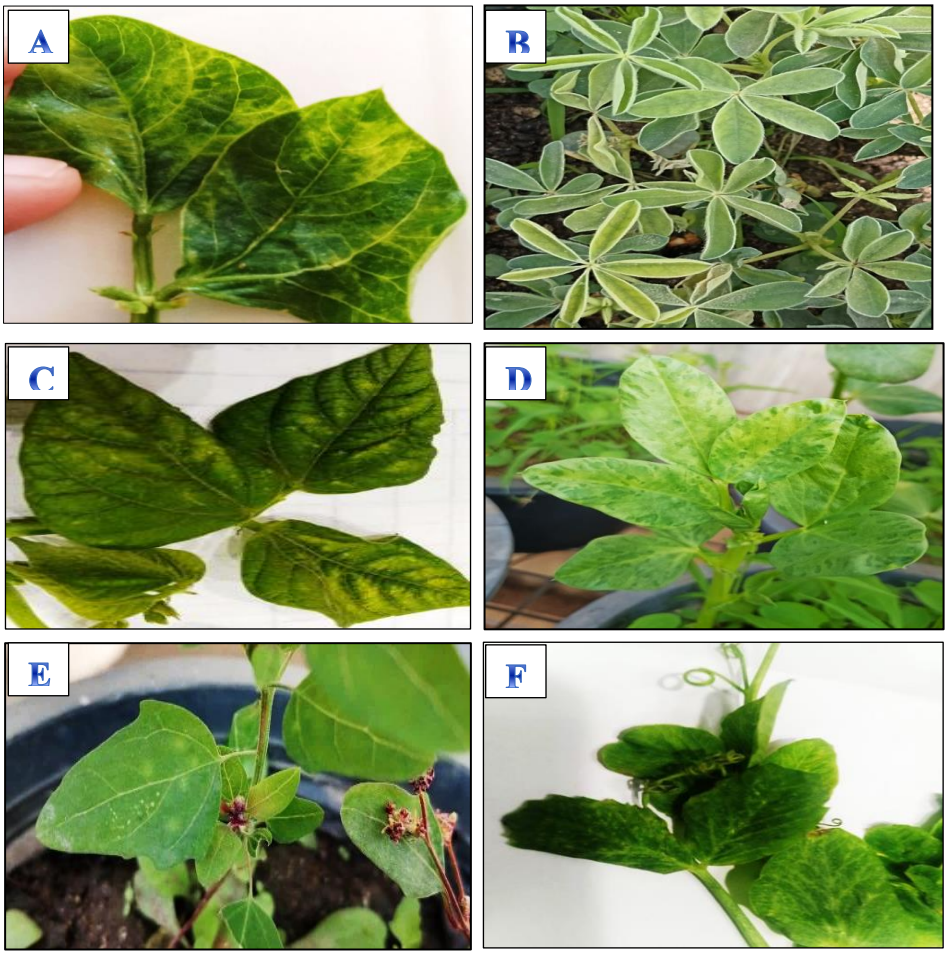

Fig. 4: Responses of some diagnostic host plants for the mechanical inoculation with BYMV. **A:** mosaic on *Vigna unguiculata,* **B:** vein greening, Leaf narrowing and plant stunting on *Lupines termis*, **C**: chlorosis on *Phaseolus vulgaris,* **D:** malformation on infected *Vicia fabae* leaves, **E:** chlorotic local lesions on *Chenopodium amaranticolor,* **F:** vein banding on *Pisum sativum.*

Detection by reverse transcription-polymerase chain reaction (RT-PCR):

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect BYMV in various tested samples. The results confirmed the specificity of the primers used in this study. The sizes of the amplification products were approximately 907 bp and 335 bp for BYMV with Specific primer BYMV-CPU, BYMV-CPD and degenerate primer U335, D335, respectively (Fig. 5. A, B).

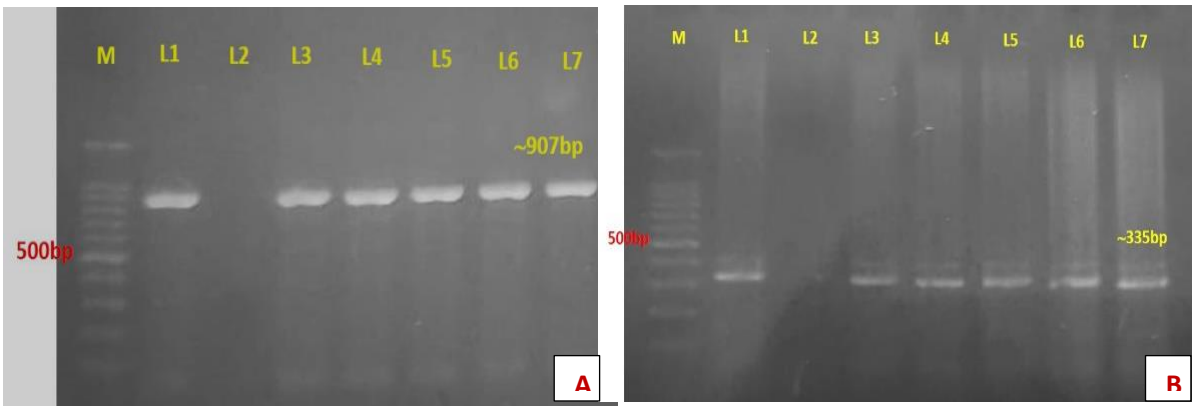

Fig. 5: (A): Agarose gel electrophoresis showing the PCR amplification products of coat protein gene of BYMV with specific primers BYMV-CPU, BYMV-CPD. M:100 bp marker, lane 1: BYMV positive control sample, lane 2: BYMV negative control sample, lanes 3, 4, 5, 6, and 7: positive samples. **(B):** Agarose gel electrophoresis showing the PCR amplification products of coat protein gene of BYMV with degenerate primers U335, D335. M: 100 bp marker, lane 1: BYMV positive control sample, lane 2: BYMV negative control sample, lanes 3, 4, 5, 6, and 7: positive samples.

Sequence analysis:

Coat protein gene nucleotide sequencing of BYMV isolate from positive purified PCR product of degenerate primers U335, D335 was submitted to GenBank database under the accession number PP481923.

Sequence analysis similarity was calculated using DNAMAN 7.0 software program (Lynnon BioSoft, Canada). The highest similarity percentage was scored between our isolate and isolates from Iran, Iraq and Mexico under accession numbers: MN241061, JQ026001 and PP098740, respectively, with a similarity percentage of 98.3%, while the percentage was 98.0% with isolates from Australia, Iran and Iraq under accession numbers: HG970867, OP525293 and JQ026005, respectively (Table 4 & Fig. 6).

Table 4. Identity percentage between BYMV under this study and other available isolates on GenBank.

Accession No.	Identity	Host plant	Country
AB041970	97.0%	Gladiolus	Japan
AB439731	96.7%	Faba bean	Japan
AM113707	90.8%	Gladiolus	India
HG970867	98.0%	Faba bean	Australia
JQ026001	98.3%	Faba bean	Iraq
JQ026005	98.0%	Faba bean	Iraq
MN241061	98.3%	Faba bean	Iran
OP525293	98.0%	Faba bean	Iran
OR233188	90.8%	Faba bean	Mexico
PP098740	98.3%	Faba bean	Mexico

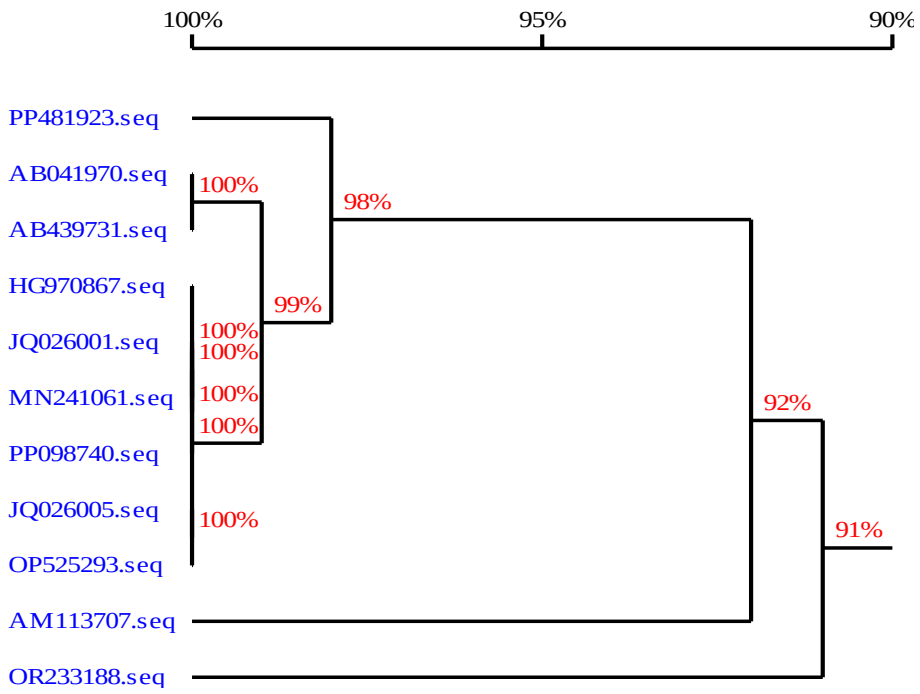

Fig. 6. A phylogenetic tree demonstrating the evolutionary relationship between our isolate (PP481923) and different isolates from the GenBank database.

Real-time RT-PCR:

Real-time RT-PCR was successfully used to determine the virus titer in all treated plants compared to the controls. The results indicated that the protective treatments were more effective than the curative treatments, with the alcoholic extraction treatment performing better than aqueous extraction treatments at both concentrations 3% and 2% while 1% in both treatments didn't show a significant difference in virus titer compared to positive control. Moreover, the peak of the melting curve was consistent across all reactions, indicating the presence of one specific product. (Fig. 7A).

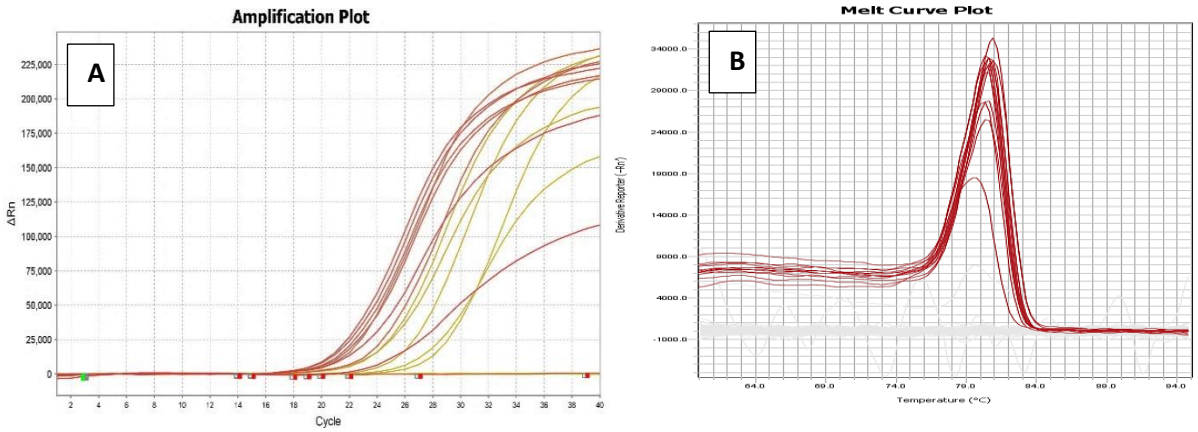

Fig. 7: The SYBR Green real-time RT-PCR assay on protective and curative treated plants with *Clitoria ternatea* extracts (Alcoholic and Aqueous). **A:** Amplification plots. **B:** The melting peaks. Each sample was tested and repeated independently three times, and Ct values above 35 were considered as a negative result.

Effect of *clitoria ternatea* flower extracts (alcoholic and aqueous) on disease incidence and severity:

Under greenhouse conditions, the untreated BYMV-mechanically inoculated faba bean plants (infected control) showed systemic symptoms, including venial yellowing, yellow mosaic, deformity, and crinkling at 15 days post-inoculation compared to those mock-inoculated with BYMV (healthy control). All different treatments with *Clitoria ternatea* extracts (alcoholic or aqueous) eliminated the effects of the virus on the faba bean plants.

Foliar application of *Clitoria ternatea* alcoholic extract in protective treatment at a concentration of 3% was the most effective treatment with a significant reduction in the percentage of infection by 100%, on the other hand, this percentage was 55.67% for *Clitoria ternatea* aqueous extract in protective treatment at 3% compared with those of the untreated infected control. Foliar application of *Clitoria ternatea* alcoholic extract in protective treatment at a concentration of 3% caused the disappearance of BYMV symptoms and resulted in a great reduction in disease severity by 100% in comparison with that in the untreated infected control. However, *Clitoria ternatea* aqueous extract in curative treatment had lower effects on reducing the percentage of infection and disease severity (Fig. 8 A, B).

Effect of *Clitoria ternatea* flower extracts (alcoholic and aqueous) on plants' growth parameters:

The viral infection showed adverse effects on faba bean plants' growth and recorded severe reduction in shoot length, leaves number, number of branches by 69.5%, 64.9%, and 82.4%, respectively, in comparison to the healthy control plants (Fig. 9 A, B and C). However, treatments with *Clitoria ternatea* extracts significantly improved these parameters, specifically that foliar application of *Clitoria ternatea* alcoholic extract in protective treatment at 3% concentration, stimulating these traits by 172%, 111%, and 233% compared to the untreated infected plants, respectively (Fig. 9 A, B and C). These growth characteristics were maximized in healthy plants treated with *Clitoria ternatea* alcoholic extract (negative control) at 3% concentration compared with the untreated healthy control plants.

In the same direction, the number of green pods per plant shoots fresh weight and shoots dry weight negatively affected by BYMV infection through inducing a significant reduction in these traits by 72.7%, 65%, and 52.6 % respectively, compared with untreated healthy control (Fig. 10 A, B and C). Exogenous application of *Clitoria ternatea* alcoholic extract in protective treatment followed by *Clitoria ternatea* aqueous extract at 3% alleviated these negative effects of BYMV infection and significantly increased the number of green pods per plant by 150% and 116.5%, shoots fresh weight by 42% and 27.3%, shoots dry weight by 31% and 28.2% compared to the untreated BYMV-infected control. Furthermore, high levels of these growth characteristics were recorded in healthy plants treated with *Clitoria ternatea* alcohol extract (negative control) at 3% concentration,

Effect of *Clitoria ternatea* flowers extracts (alcoholic and aqueous) on photosynthetic pigments:

The presented data in Figure 11 showed a significant reduction in the photosynthetic pigments of untreated BYMV-infected control. The chlorophyll a, b and total chlorophyll content decreased by 79%, 35.5%, and 56.8%, respectively, as compared to healthy plants. However, The treatment of BYMV-inoculated plants with *Clitoria ternatea* alcoholic extract greatly enhanced photosynthetic pigments, particularly protective treatment at 3% increased the chlorophyll a, b and total chlorophyll content by 248.6%, 66.6%, and 76.3% followed by 237.5%, 52.2%, and 62.2% for *Clitoria ternatea* aqueous extract in protective treatment at 3%, over those of the untreated infected control.

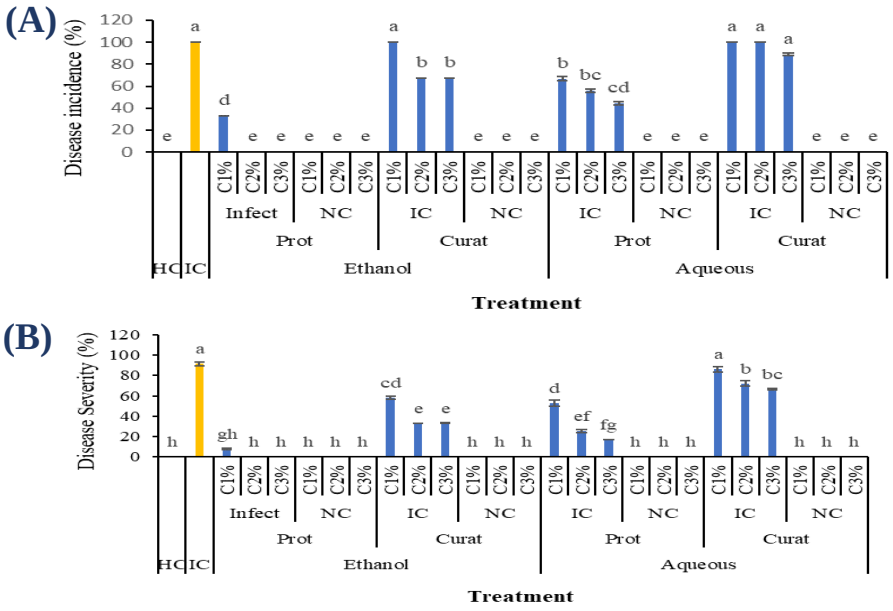

Fig. 8: The impact *Clitoria ternatea* flowers extracts (alcoholic and aqueous) foliar application on *Bean yellow mosaic virus* (BYMV), disease incidence (A) and disease severity (B). HC= healthy, IC= infected control,

NC= negative control, C= concentration, Prot= protective treatment, Curat= curative treatment. Means followed by the same letter in each column were not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

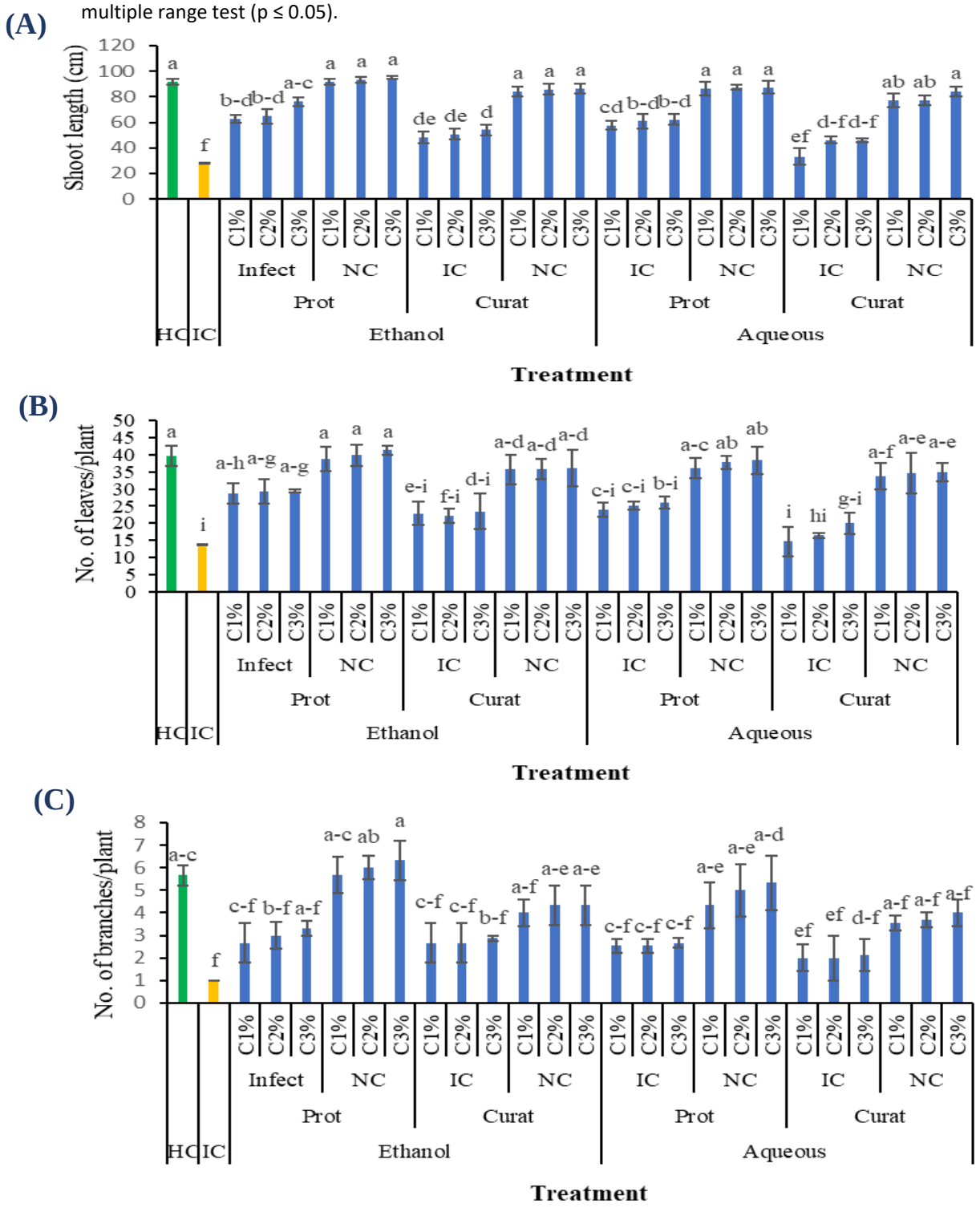

Fig. 9: The impact of *Clitoria ternatea* flowers extracts foliar application on faba bean growth characteristics. (A) shoot length, (B) No. of branches plant^{-1}, (C) under *Bean yellow mosaic virus* (BYMV) infection. HC= healthy, IC= infected control, NC= negative control, C= concentration, Prot= protective treatment, Curat= curative treatment. Means followed by the same letter in each column were not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

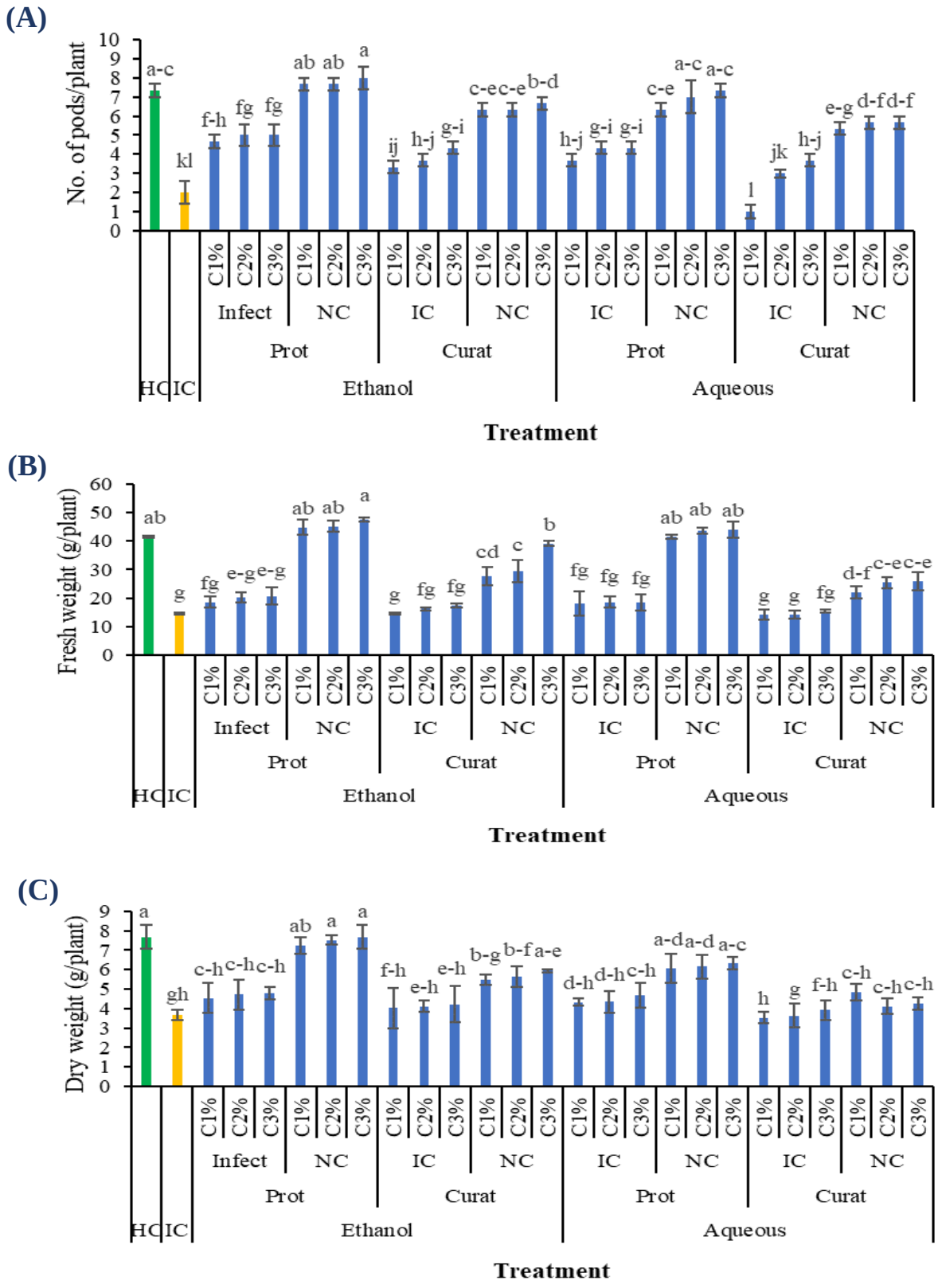

Fig. 10: The impact of *Clitoria ternatea* flower extracts foliar application on No. of pods and biomass of faba bean inoculated with BYMV. (A) No. of pods plant^{-1}, (B) plant fresh weight, and (C) plant dry weight). HC= healthy, IC= infected control, NC= negative control, C= concentration, Prot= protective treatment,

Curat= curative treatment. Means followed by the same letter in each column were not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

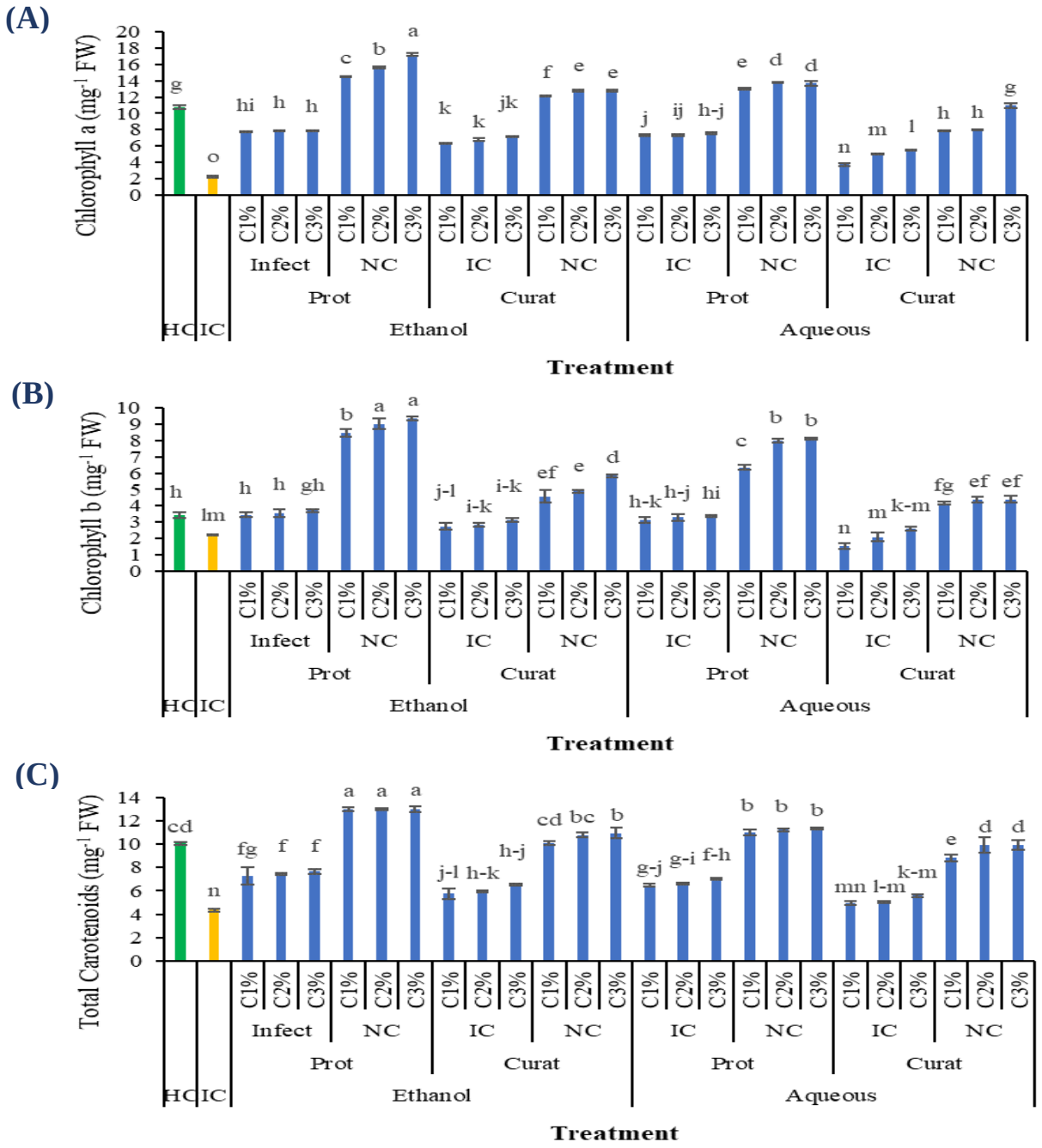

Fig. 11: The impact of *Clitoria ternatea* flowers extracts foliar application on photosynthetic pigments. (A) chlorophyll *a*, (B) chlorophyll *b*, and (C) total carotenoids of faba bean plants inoculated with BYMV. HC= healthy, IC= infected control, NC= negative control, C= concentration, Prot= protective treatment, Curat= curative treatment, FW= fresh weight. Means followed by the same letter in each column were not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

DISCUSSION

Bean yellow mosaic virus (BYMV) is one of the most important viral diseases in many regions worldwide. BYMV has been isolated from legumes from Africa, Asia, Australia, Europe, North America and South America (Kumari and Makkouk 2007; Kehoe *et al*., 2014; Lisa 2000; Rashed *et al*., 2018 and Campos *et al*., 2013). This study demonstrated the distribution of the virus in four governorates (Fayoum, Beheira, Giza, Bani Seuf). RT-PCR

detection showed positive results for 54 out of 95 samples. The results proved the existence of the virus in all faba bean growing areas.

The incidence data illustrated the widespread infection of BYMV in major faba bean growing districts with various incidences. The distribution of BYMV can vary, depending on factors such as agricultural practices, cropping systems, and the presence of suitable hosts and vectors. The virus can spread locally within fields through aphid movement, as well as over longer distances through the transportation of infected plants or seeds. In the future more samples must be tested from different governorates to understand the incidence and distribution of the virus. Previous studies reported the presence of BYMV in different governorates of Egypt such as Ismailia (El-Bramawy and El-Beshehy 2012), Qalyubia (Mahdy *et al.,* 2007), Gharbia (El-Sayed *et al*., 2019), Beheira (Younes *et al*., 2021) and Menoufia (El-Helaly *et al*., 2016). Host range study revealed that 8 out of 16 tested plant species belonging to the family *Leguminosae* and *Chenopodiaceae* were infected and showed symptoms varied among different host plants. These symptoms have been previously documented in several studies (Bos 1970; Radwan *et al*., 2008 and El-Helaly *et al.*, 2016).

RT-PCR Amplification confirmed the presence of BYMV, RT-PCR revealed amplification of 907 bp with specific primers for coat protein (CP) gene and 335 bp using degenerate primers for potyvirus and these results agreed with Langeveld *et al.* (1991), El-Helaly *et al*. (2016) and Mohamad *et al*. (2008). Phylogenetic analysis for the partial sequence of BYMV coat protein gene-coding region (335 bp) demonstrated that the isolate in this study had an identity ranging from 90.8% to 98.3% compared with ten BYMV sequences available on GenBank.

Infection with BYMV has significant impacts on the growth parameter, physiological processes and development of infected plants (Radwan *et al*., 2008; El-Helaly *et al*., 2016 and Kaur *et al*., 2024). Control of plant viral diseases can be accomplished by inducing plant defence mechanisms, e.g., systemic acquired resistance (SAR) (Ryals *et al*., 1994). Certain plant extracts were used against BYMV infection on faba bean plants and it was promoted to induce systemic resistance (Elsharkawy *et al*., 2021).

Plant extracts containing bioactive agents that found to be useful as antibacterial, antifungal and antiviral, the phytochemicals that have antiviral effects include various flavonoids, terpenoids, alkaloids and polyphenols, each of which exhibit various therapeutic effects during different stages of the viral cycle (e.g. viral attachment, entry, viral replication, and release) (Ghildiyal *et al*., 2020). Based on the previous studies that reported the benefits of Butterfly pea flower as a human antiviral (Nugraha *et al*., 2021; Fazadini and Yzzuddin 2022; Torres *et al.,* 2022 and Chun *et al*., 2023), accordingly this investigation attempted to use flower extract to control BYMV infection.

Under greenhouse conditions, the effect of two foliar application times (protective and curative treatment) was evaluated in three concentrations of extract (1%, 2% and 3%) and two methods of extraction (ethanol alcohol and water) on the disease severity, growth parameter, photosynthetic pigments and virus concentration. The results revealed that the most effective treatment was 3% concentration in the protective treatment whether extracted using ethanol or water. Treatments with extracts significantly improved all studied parameters, these parameters were maximized in healthy plants treated with alcohol or water *Clitoria ternatea* extract (negative control) at 3% concentration compared with the untreated healthy control plants. The treatment with 3% concentration in the protective treatment was able to eliminate the infection, and 100% of plants that did not show any symptoms were obtained. On the other hand, the curative treatment at the same concentration showed high incidence and severity.

The bioactive compounds in both extracts were not determined, but based on the literature the bioactive compounds reported to be found in the flowers are flavonols (quercetin, myricetin, and kaempferol derivatives) and anthocyanins (ternatin A1-A3, B1-B4, C1-C4, and D1-D3) (Kazuma *et al*., 2003). This effective bioactive extract is affected by various factors, including the solvent concentration, extraction time, temperature, and solvent/material ratio of the extraction (Pham *et al*., 2019 and Jeyaraj *et al*., 2021), and hence the findings in this study make sense. Anthocyanins are the largest group of water-soluble pigments belonging to flavonoids, a subclass of the polyphenol family (Jing & Giusti 2011 and Gupta & Chakrabarty 2016). The flavonoids in the extract are the primary contributors to the antioxidant activity of *Clitoria ternatea*. Therefore, *Clitoria ternatea* can be considered as a good source of antioxidants, which may be beneficial in preventing the progression of various oxidative stress-related conditions (Torres *et al.,* 2022). Moreover, *C. ternatea* active compounds of anthocyanin and ternatin support the immune system where they act as immunoregulatory, antioxidant properties of bioactive compounds play a major role in immunoregulation activity (Nugraha *et al*., 2021).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a widely used technique for detecting and quantifying RNA, including viral RNA in plant samples. This method is crucial for understanding viral load, gene expression, and the effectiveness of antiviral treatments in plants (Liapunova *et al*., 2019). In the present work, real-time RT-PCR determined the titer of the virus in all treated plants compared with

controls. Results were in the same direction as reported in the growth parameter that a low concentration of virus was in protective alcoholic treatment at 3% concentration, this result confirmed the suggestion that *Clitoria ternatea* flower extract stimulates resistance mechanisms (induces resistance), rather than having direct antiviral properties.

CONCLUSION

In this study, the incidence of BYMV in different locations across four governorates in Egypt. Typical symptoms of the virus were observed in faba bean fields, with incidence rates ranging from 14.4 to 46.4%. The presence of the virus in symptomatic samples was detected by RT-PCR. Sequence comparisons of the BYMV coat protein gene revealed similarities ranging from 90.8% to 98.3% with ten reported isolates of BYMV compared to the Egyptian isolate. This study provides evidence for the efficiency of *Clitoria ternatea* flower extract in triggering the faba bean plant's immune system against BYMV infection by reducing disease incidence and severity and improving growth parameters and photosynthetic pigments.

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