Resistance Induction in Two Alfalfa Varieties to Bean Leafroll Virus and Citrus Excortis Viroid Using Some Botanicals and Amino Acids Preparations

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ABSTRACT
Many researches have been conducted so far in the topic of controlling the Bean leafroll virus and Citrus exocortis viroid. This study was conducted in a private farm at Balad district from September 1, 2023, to January 6, 2024, and aimed to evaluate the efficacy of azolla and clover plant extract, fosetyl aluminum pesticide, and Debal against Bean leafroll virus in the presence of Citrus exocortis viroid in diseased alfalfa. The diagnosis was confirmed for the whole genome of the virus and viroid under study using next generation sequencing technology. The resulting sequences were compared in the International Gene Bank database NCBI. The virus was the 1st record registered in Iraq, which was registered with a accession number PP889325, and the viroid genome was registered for the first time in the NCBI database with an accession number PP869624. Findings of the field experiment showed that the treatments were effective in reducing infection rates and disease severity beside increasing plant defense enzymes (catalase, polyphenol oxidase, and peroxidase) in virus infected alfalfa. Debal treatment, individual or in combination, recorded the highest rate leaf content of catalase (8.00), polyphenol oxidase (3.70), and peroxidase (4.13), with values significantly differed from infected untreated control treatment that recorded 3.33, 0.91, and 0.81, respectively.

1. Introduction

Alfalfa (*Medicago sativa* L.) belongs to the *fabaceae* family (16), and most specialists believe that members of *fabaceae* family have grow in arid and semiarid regions (33). Alfalfa plants are perennial forage herbs and quality characteristics for forage legumes depends on field crop practices, cultivar, soil fertility, harvest date, environment conditions especially temperature and moisture, the storage (18). The plant is considered as an important host for a wide range of plant viruses such as *alfalfa mosaic virus* (AMV) (*genus Alfamovirus, family Bromoviridae*), *Cucumber mosaic virus* (CMV) (*genus Cucumovirus, family Bromoviridae*) *and bean leafroll virus* (BLRV) *genus Luteovirus, family Luteoviridae*) which are the most common viruses in alfalfa fileds (13). BLRV Argentina showed a wide distribution of over 50% in Argentine alfalfa fields, and genome of the isolate *manfredi BLRV-Arg* is 5884 nucleotides in length (38). With the aid of high throughput sequencing, the BLRV is detected to be an RNA virus (6). The mean survival rate and rate of population growth of pea aphid *Acyrthosiphon pisum* were increased by 15% and 14%, respectively as a result aphid feeding on alfalfa plants infected with BLRV, this reflects the economic importance for virus transmission and epidemic (9). Also *Aphis fabae* acquire the virus from infected plants (28), *Citrus excortis viroid* (CEVd) is economically important (30) which belongs to the family Pospiviroidae, in the genus Pospiviroid (32). Symptoms of infection by CEVd are in a form initial bark shelling and produces subsequent sloughing symptoms on *Poncirus trifoliata*, rangpur lime and troyer citrange (12). CEVd diagnosed in Taiwan has a body length of 365–475 nt (17), which was also discovered on lettuce plant *Lactuca sativa* L. In Iraq, a complete genome length for Citrus excortis viroid isolate was to be 394 nt (4). *Azolla microphylla* are a good source of proteins, essential minerals and vitamins (8) and contains calcium and phosphorus in proportions of 1.32% and 0.86%, respectively (35). There is a symbiotic coexistence that fixes atmospheric nitrogen between the nitrogen-fixing cyanobacterium *Anabaena azollae* and the Azolla species *A. microphylla*, as this symbiosis is of a permanent nature. Adding humic acid in different form of fertilizers (39), seems to have many positive effects of plant growth and plant physiological activities including photosynthesis and increasing absorption of ammonia due to its increased representation within the plant (14). Another product is the compound Fosetyl-aluminium, fosetyl-Al, is a highly polar fungicide that is commonly used as an alternative to sodium arsenite, which is banned globally (7). Fosetyl-Alminum 80%WG is an organic systematic broad-spectrum protectant pesticide against wide range of fungal and bacterial plant pathogens (33). This research work aimed to investigate possibility of designing a multi-factors management strategy to control *Bean leafroll virus*

in the presence of *Citrus excortis viroid* and reduce their effect in diseased alfalfa using azolla and clover plant extract, Fosetyl-aluminum pesticide, and/or Debal individually or combined treatment.

2. Methodology

The experiment factors

In case azolla extract, 1 kg of Azolla was brought from one of the nurseries in Balad district, planted in 50x50 cm culture trays containing water media and maintained in covered protected shade place. Quantities of Azolla were taken and inserted into the press machine to make an extract with a concentration of 100%. **For Clover extract,** The clover plants were collected from farms in Balad district, as the stems and leaves were used, placed into the food processer to obtain a plant extract with a concentration of 100%. **Regarding to Debal (Humic acid),** this product is mainly humic acid 12% and organic base of 49%, produced by Jana Co. for fertilizers used at rate 1.5 ml/L water. Which was obtained from Al-Najah market for agricultural materials. **To be used as chemical comparison, Fosetyl aluminum chemical pesticide was** brought from one of a certified agricultural market in Balad district.

Virus and viroid detection

Immunological strips were used to detect infected secondary hosts as Immuno-strips containing an anti-gene mosaic virus serum prepared by the American company Agdia. The examination was performed according to the method recommended by the supplied company. 3-5 plant samples (leaves) taken from some economic crops and weed plants were examined. The presence of the virus in the hosts was confirmed, and the infected juice was later used for inoculating the experimental plants.

Virus molecular confirmation

(20) were followed in preparing RNAlater to preserve RNA and DNA from being destroyed, and it was sent to DNA Link Inc Republic of Korea for the purpose of reading the complete sequence of total RNA and total DNA in the infected sample according to the method of work followed by the company. This company produces sequences in the form of raw reads with a length of 101 base pairs for RNA and 150 base pairs for DNA. The total RNA sequence was read according to the specifications approved by the company DNA Link Inc Republic of Korea using the program NovaSeq6000, 101 Baired End and the Trueseq RNA method approved by the company. The total DNA sequence was also read according to the following specifications (X 150 PE (2) (NovaSeq 6000), according to the manufacturer's method of producing sequences in the form of raw reads 150 base pairs long.

Experiment General Procedure

This experiment was carried out in a private farm in Balad District on September 1, 2023. Where alfalfa seeds of two varieties 'Zwabaa' local and the Indian imported KDR were sewn in 150x100 cm plots with three replications for each treatment, 33 plot in total, as the results were obtained on January 6, 2024. For the artificial virus infection, A phosphate extraction buffer solution was prepared using Na2HPO4 1.42g/L and B- KH2PO4 1.362g/L, Mixing 51 ml of solution A with 49 ml of solution B to obtain a concentration of 1.01 molar and pH= 7 (30,5). Then, 4 ml of cooled phosphate extraction buffer solution was added to every 1 gram of alfalfa leaves infected with the virus, crushed well using a ceramic mortar, then the extract juice was passed through gauze and the filtrate was approved for infection. The infection was carried out on the alfalfa plant varieties on May 25, 2023, where the leaves of the plants were sprayed with carborundum (600 mesh) to create wounds in the leaf and facilitate the entry of virus particles (32). Then the leaves of the plants were wiped with the viral inoculum, after which the inoculated plants were sprayed with distilled water after 1 - 2 minutes of infection.

The experiment units were distributed in Randomized Complete Block Design (RCBD), alfalfa seeds have been planted, upon reaching vegetative growth stage, artificial infection with the virus was

performed on 11-1-2023. The prepared plant extracts and chemicals were sprayed according experiment treatments. Treatments were

Plant extracts of azolla or clover, Debal, and fosetyl aluminum pesticide and all the interactions beside the control sprayed with DW to control the Bean leafroll virus in presence of Citrus excortis viroid in alfalfa a local and imported varieties. The plants were sprayed with an amount of 2 ml/plant of each factor. the results were taken during the period between 11-6-2023 and 1-6-2024.

Measurements under study

Infection rate and severity

Symptoms appeared on the cultivated puffer plant Alfalfa two weeks after the artificial infection was carried out, and the infection rate was calculated. As for infection severity, it was calculated using a 6-degree disease scale designed based on disease symptoms appeared on plant leaves where: 0 = intact leaf, 1 = spots, 2=yellow spots, 3=Severe mosaic spots on leaves, 4=Severe yellowing of leaves, 5 = yellowing, distortion, and reduction of leaf area. (23) equation was applied to calculate the injury (infection) severity.

Defense enzymes measurements

Polyphenol oxidase enzyme was estimated in plant leaf using a spectrophotometer-UV at a wavelength of 470 nm (22). As for **peroxidase enzyme** determination, it was determined by Coycol method using 0.1 ml sample volume, as 0.01 is one unit of enzyme (the amount of enzyme that causes an increase in light absorption of 0.1 units per minute at a wavelength of 420 nm (24). **Catalase enzyme was estimated** depending on amount of change in absorbance at a wavelength of 240 nm for a 30 mmol hydrogen peroxide solution in the presence of phosphate buffer solution contains K_2HPO_4, KH_2PO_4, H_2O_2 (2).

Statistical Analysis

The experiment data were analyzed by GenStat 12th software (38), and the averages were compared according to the least significant difference L.S.D. at or below the probability level (0.05) (3).

3. Results and discussion

The diagnosis was confirmed for the whole genome of the virus (Bean leafroll virus) and the viroid (Citrus exocortis viroid) under study using next generation sequencing technology. The resulting sequences were compared in the International Gene Bank database NCBI. The virus was the 1st record registered in Iraq, which was registered with a accession number PP889325, and the viroid genome was registered for the first time in the NCBI database with an accession number PP869624.

The results showed that the different treatments (Table 1) had a clear effect on the infection rate, and the infection rate differed between the two varieties used. Except for the control treatment, the highest infection rate of 60% among the treatments was recorded in the azola treatment of the local variety, which also decreased from the infection rate recorded in the control treatment of 87%. While the lowest infection rate for the two types used was recorded in the interaction treatment between Azola and Debal, 22% in the local variety and 15% in the imported variety, with a significant difference from the interaction treatments, even in the presence of the pesticide. In general, the infection rate of the imported variety was significantly lower than the local variety for all treatments. In the same way, the results show that the severity of infection was also higher in the local variety than the imported variety, regardless of the type of treatment (Table 1). The azola debal treatment also recorded less infection severity in the local variety 1.3, which did not differ from the Debal treatment with the pesticide. All intervention treatments recorded lower infection severity rates than the individual treatments, and all treatments led to a significant reduction in infection severity over the untreated control. Similar results, but with significantly lower values, were recorded on the imported variety, which showed a

significantly lower infection severity in the single and overlapping Debal treatments compared to the other treatments (Table 1).

Table1. Effect of the experiment treatments on infection rate and severity in two alfalfa varieties caused by Bean leafroll virus in presence of Citrus exocortis viroid

Treatments	Infection rate % on alfalfa		Infection severity	
	LOCAL	Imported	LOCAL	Imported
Azola	60%	53%	2.7	2.7
Clover	52%	38%	3.0	2.7
Debal	35%	18%	2.3	1.0
Pesticide	55%	47%	3.0	2.7
azola + clover	38%	22%	2.3	1.3
azola + debal	22%	15%	1.3	1.0
azola + pesticide	55%	43%	3.0	2.3
clover + debal	45%	20%	2.3	1.0
clover + pesticide	45%	20%	2.7	1.7
debal + pesticide	35%	25%	1.3	1.0
control 1	87%	82%	4.7	4.3

Efficacy of azolla and clover extracts, debal, and fosetyl aluminum in controlling Bean leafroll virus and Citrus exocortis viroid in two alfalfa varieties

The results of Table (1) related to the effect of treatments on effectiveness of catalase enzyme showed that there were significant significant effects, as all of the studied treatments were significantly superior to comparison treatment by increasing the effectiveness of the enzyme, as pesticide with debal treatment gave the highest effectiveness rate of 8.00, with large significant differences from the comparison treatment. Which gave lowest effectiveness rate of 3.33, but it did not differ significantly from the rest of the other treatments. As for plant varieties, no significant differences were recorded, as the imported and local variety gave an enzyme effectiveness rate of 7.29. As for the total interaction between treatments and plant varieties studied, there were significant differences recorded, as Debal + pesticide treatment to imported variety gave the highest enzyme effectiveness rate of 8.37 compared to comparison treatment of the imported variety, which recorded lowest rate of 3.40, and Clover treatment of the local variety gave the highest effectiveness rate. It reached 7.83 compared to comparison treatment for the local variety, which recorded lowest rate of 3.27.

Increasing the effectiveness of the catalase enzyme in the plant as a result of applying the aforementioned treatments to the plant is a sure reflection of the stimulation of the plant's defense mechanisms against the virus, as it was found that the catalase enzyme works to activate and strengthen the immune defense methods and produce resistance-specific antibodies in the infection area of the tobacco plant infected with the Tobacco mosaic virus TMV (36). In TMV, the catalase enzyme synthesizes proteins for acquired resistance (ASR) in order to enhance the activity of acquired resistance while increasing root secretions that help reduce the severity of the infection (36). The catalase enzyme is also closely linked to the genetic expression of genes that enhance resistance in tobacco plants infected with the TMV virus (19), and the mechanism of inducing systemic resistance (ISR) depends on signals from the synthesis of jasmonic acid (JA) and ethylene (ET) to activate the plant's defenses (19). It was also indicated (1) that adding some biological factors to the cowpea plant 4 days before mechanically inoculating it with Cowpea mild mottle virus (CPMMV) gave the highest content of the catalase enzyme, amounting to 9.86, while adding biological factors to the plant after 4 days. Days after mechanical inoculation gave an enzyme content of 7.18 compared to the control treatment of 4.04, attributing this to stimulating the plant's defenses against the virus.

The results of Table (2) related to effect of treatments on effectiveness of polyphenol oxidase enzyme

showed that there were significant effects, as all of the studied treatments were significantly superior to comparison treatment by increasing effectiveness of the enzyme, as debal treatment gave highest effectiveness rate of 3.70, with significant differences from comparison treatment. Which gave lowest effectiveness rate of 0.91, but it did not differ significantly from the rest of other treatments. As for plant varieties, significant differences were recorded, as the imported variety gave highest enzyme effectiveness rate of 3.41, while the local variety gave 2.83. As for total interaction between treatments and plant varieties studied, there were significant differences recorded, as clover + Debal treatment gave imported variety highest enzyme effectiveness rate of 4.03 compared to comparison treatment of the imported variety, which recorded lowest rate of 0.96, clover + pesticide treatment gave to the local variety highest activity rate of enzyme was 3.66 compared to comparison treatment for the local variety, which recorded lowest rate of 0.86.

Table 2. Effect of different treatments spray on Catalase enzyme effectiveness in leaf of two alfalfa varieties infected with Bean leafroll virus and Citrus exocortis viroid

Treatments	**Catalase enzyme**		**treatments average**
	Local	Imported	
Azola	6.57	6.97	6.77
Clover	7.83	6.83	7.00
Debal	6.60	7.03	6.82
Pesticide	7.63	7.50	7.57
azola + clover	7.50	7.33	7.42
azola + debal	7.67	7.67	7.67
azola + pesticide	7.53	7.67	7.60
clover + debal	7.50	7.50	7.50
clover + pesticide	7.30	7.67	7.48
debal + pesticide	7.63	8.37	8.00
control 1	3.27	3.40	3.33
varieties average	7.29	7.29	7.29
L.S.D 0.05	0.65 varieties		1.85 treatments
	2.62interaction		

An increase in the polyphenol enzyme is an indication of the activation of systemic resistance in plants against viroid or viral pathogens, as it hinders the development of the disease in the plant (11). The reason for the increase in enzyme production may be due to the role of the control agents used in the study, such as humus extract, in stimulating the plant to On the production of phenolic content in large quantities, and this is consistent with what was mentioned by Abdul (26), This could also be due to the fact that plants infected with the virus increase the amount of oxidative enzymes in the cells when they are exposed to the infection, especially if the infection is reinforced several times, which indicates their resistance to the virus. This was confirmed by Wood in 1899 when he noticed an increase in oxidative enzymes in tobacco plants infected with the mosaic virus. Tobacco (27), and (4) indicated that the roots and leaves of lettuce plants infected with Citrus exocortis viroid gave the highest significant difference in the effectiveness of the polyphenol enzyme, as the infected local variety outperformed the healthy local variety in Enzyme effectiveness. It was found that the activity of the polyphenol oxidase enzyme increased in the leaves of Capsicum annuum pepper plants after inoculation with Geminivirus, which causes leaf curl disease, compared to the leaves of non-inoculated plants (28).

Table 3. Effect of different treatments spray on Polyphenol oxidase enzyme effectiveness in leaf of two alfalfa varieties infected with Bean leafroll virus and Citrus exocortis viroid

Treatments	**Polyphenol oxidase enzyme**		**treatments average**
	Local	**Imported**	

Azola	2.73	3.33	3.03
Clover	3.00	3.56	3.28
Debal	3.60	3.80	3.70
Pesticide	3.06	3.60	3.33
azola + clover	3.16	3.93	3.55
azola + debal	2.80	3.60	3.20
azola + pesticide	2.70	3.43	3.06
clover + debal	3.20	4.03	3.61
clover + pesticide	3.66	3.33	3.50
debal + pesticide	2.40	4.00	3.20
control 1	0.86	0.96	0.91
varieties average	2.83	3.41	3.12
L.S.D 0.05	**varieties 0.39**		**treatments 1.12**
	1.59 interaction		

The effectiveness of peroxidase enzyme was also significantly different among treatments, as all of the studied treatments were significantly superior to comparison treatment by increasing enzyme activity. The pesticide treatment gave highest effectiveness rate of 4.13, with significant differences from comparison treatment that gave lowest effectiveness rate was 0.81, but it did not differ significantly from the rest of other treatments. As for plant varieties, significant differences were recorded, as imported variety gave highest enzyme effectiveness rate of 3.91, while the local variety gave 3.31. As for the total interaction between treatments and plant varieties studied, there were significant differences recorded, as azola + pesticide , debal+pesticide treatments in imported alfalfa gave highest enzyme effectiveness rate of 4.43 compared to the control of the same variety, which recorded lowest rate of 0.86. The pesticide treatment for the local variety had highest enzyme effectiveness rate of 3.96 compared to 0.76 in the control.

The peroxidase enzyme catalyzes the oxidation of hydrogen-donating materials in the presence of hydrogen peroxide, H2O2, to produce compounds that are toxic to pathogens. The enzyme also oxidizes phenolic compounds to more toxic substances called quinones, which may work to inhibit the virus by breaking down the proteins it needs in the replication process, or it directly affects the virus. The virus protein, in addition to its role in stimulating the plant's systemic resistance to the virus (15), This study is consistent with many studies that indicated an increase in the effectiveness of the peroxidase enzyme in plants as a result of inducing plant resistance (therapeutically or preventively) by biotic or abiotic factors against pathogens. It was found that the effectiveness of the peroxidase enzyme increased in the leaves of Capsicum annuum pepper plants after... Inoculated with Geminivirus, which causes leaf curl disease, compared to the leaves of uninoculated plants (28), enhanced phenolic and peroxidase synthesis in different host groups is associated with disease resistance (15).(1)stated that adding biological factors to the cowpea plant achieved the highest rate of the peroxidase enzyme in the plant, which reached 23.73 four days before mechanical infection with Cowpea mild mottle virus, and it reached 21.60 four days after infection, compared to the control treatment, which reached 16.83. Before and four days after the infection. The activity of the peroxidase enzyme increased in banana varieties infected with the Banana bunchy top virus, and its activity was linked to the resistance reaction, which could be due to an increase in the concentration of phenol, as phenols are the cofactor for peroxidase and thus affect the stimulation of resistance in the host (10).

Table 4. Effect of different treatments spray on Peroxidase enzyme effectiveness in leaf of two alfalfa varieties infected with Bean leafroll virus and Citrus exocortis viroi

Treatments	**Peroxidase enzyme**		**treatments average**
	Local	**Imported**	
Azola	3.16	4.26	3.71

Clover	3.80	3.83	3.81
Debal	3.36	3.76	3.56
Pesticide	3.96	4.30	4.13
azola + clover	3.60	4.36	3.98
azola + debal	3.66	4.30	3.98
azola + pesticide	3.20	4.43	3.81
clover + debal	3.53	4.26	3.90
clover + pesticide	3.80	4.26	4.03
debal + pesticide	3.66	4.43	4.05
control 1	0.76	0.86	0.81
varieties average	3.31	3.91	3.61
	0.32 varieties		**0.90 treatments**
L.S.D 0.05	**1.28 interaction**		

4. Conclusion and future scope

We conclude from the current study that extracts which included plant extract of azolla and clover, fertilization with Debal, and fosetyl aluminum pesticide application, have an important role in inhibiting the Bean leafroll virus existence with Citrus excortis viroid in diseased alfalfa. This indicates the possibility of including botanicals and commercial fertilizers within integrated management programs for controlling viroid and viral infections.

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