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Research Article

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# Molecular Detection of Bean Yellow Mosaic Virus in *Lupinus albus* Plants and its Associated Alterations in Biochemical and Physiological Parameters

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## Abstract

Bean yellow mosaic virus is one of the most devastating diseases of cultivated Leguminosae plants worldwide causing mosaic, mottling, malformation and distortion in infected cultivar plants. Present study was conducted to investigate the possibility of infection of *Lupinus albus* (Lupine) with Bean yellow mosaic virus. Virus isolate was identified by detection of the coat protein gene amplified by reverse transcription polymerase chain reaction and also *via Chenopodium Amaranticolor* as a diagnostic host plant. Results showed that infection can be induced under greenhouse conditions and infected plants showed a considerable level of mosaic symptoms. As disease development in infected plants is always associated with physiological and chemical changes, some metabolic alterations parameters have been evaluated like photosynthetic pigment contents, total carbohydrate content, total soluble protein, total protein, total free amino acid, proline induction, total phenolics, salicylic acid, and abscisic acid content in healthy and infected lupine plants. Results showed a great variation in all the biochemical categories in *Lupinus albus* infected with bean yellow mosaic virus as compared to healthy plants. Chlorophyll a of virus inoculated *Lupinus albus* decreased to 27%, whereas Chlorophyll b content decreased to 19.5% and carbohydrate content decreased to 36% when compared to healthy control plant corresponding values. Results also showed many metabolic changes in virus infected Lupine plants. The effect of virus infection on the induction of plant growth regulators like abscisic acid was determined, as well as the relationship between abscisic acid activation, accumulation of the virus, and symptoms development was discussed, and the effect of abscisic acid inhibitor application on virus infection and Lupine primary and secondary metabolism was elucidated, as this effect is a neglected field of research.

**Keywords:** Lupine; Antiviral; Bean yellow mosaic

## Introduction

*Lupinus albus* is an alternative to soybean for the production of a high protein crop in temperate climates. It is more tolerant to low soil pH and low temperature than soybean. Bean yellow mosaic virus (BYMV), a member of the potyvirus family [1], is transmitted to lupine plants by aphids and through seed [2]. BYMV has a wide host range that includes most leguminous plants. Infected white lupine plants display narrower leaflets, vein clearing, necrotic spotting, severe mosaic and leaf deformation. Early infection results in severe stunting and bushy appearance of plants. The virus was first reported by Pierce (1993) [3]. BYMV is not seed-borne in French bean but transmitted to a small percentage in luteus, Metilotus alba, Pisum sativum, and *V. faba* [4,5].

Plant hormones are small signaling molecules that regulate growth and development, although they exist in small amounts, changes in their concentrations affect adaptive plant responses to biotic or abiotic stresses Mazen et al., [6]. Plant responses to biotic stress are controlled by the following hormone-regulated defense pathways: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) [7]. These pathways antagonize one another and, thus, plants can adjust the levels of interaction to maintain effective defense while minimizing the metabolic cost. Plant resistance to viruses is usually mediated by the SA pathway. Upon activation, this pathway induces several defense responses, including reactive oxygen species (ROS) production, the hypersensitive response, and pathogenesis-related (PR) protein synthesis [8]. Salicylic acid (SA) or ortho-hydroxy benzoic acid and related compounds belong to a diverse group of plant phenolics. Salicylates from plant sources have been used in medicine for a long time. Using modern analytical techniques it has been found that salicylates are distributed in many important agricultural plant species. SA is a phenolic phytohormone playing a role in plant growth and development, photosynthesis, transpiration, ion uptake and transport. SA also induces specific changes in leaf anatomy and chloroplast structure. SA is involved in endogenous signaling, mediating plant defense against pathogens. It plays a role in the resistance to pathogens by inducing the production of pathogenesis-related proteins [9]. The key hormone in the response to abiotic stress is abscisic acid (ABA). The effect of ABA on plant disease resistance is a neglected field of research [10]. ABA strongly antagonizes many hormone pathways, including SA [11], ET [12], and the synergistic Et/JA pathway [13]. The role of ABA in plant defense was shown to be phase specific. Although the interactions between ABA and bacterial or fungal pathogens have been thoroughly studied, ABA–virus interactions are weakly analyzed [6]. Several studies showed that ABA is induced upon several viral infections but only a few works focused on how ABA is involved in plant defense to viruses. AS indicated in some studies, ABA content was not affected in potato cultivars infected with Potato virus Y [14]. ABA levels were also increased in nonheading Chinese cabbage infected with Turnip mosaic virus [15].

ABA plays a central role in the response and adaptation to

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environmental constrains. However, apart from the well-established role of ABA in regulating gene expression, little is known about its function in plant stress metabolism [16]. ABA is also important for many aspects of plant growth and development, including the control of gas exchange, seed development and germination, triggering adaptive changes occurring in response to abiotic stressors.

Although ABA plays a key role in modulating plant responses to different biotic and abiotic stresses, its effect on virus infection is not fully understood. Here, the impact effects of BYMV infection on primary and secondary metabolic processes of Lupine plants, like photosynthetic, pigment content, total carbohydrates, total proline, total soluble protein, total amino acid, total phenolics, SA and ABA content besides the effect of endogenous ABA on the primary and secondary metabolism and infection process.

The effect of ABA inhibitor, nordihydroguaiaretic acid (NDGA) treatment will also be studied for the first time on the primary and secondary metabolism of the infected Lupine plants as well as its effect on the infection with BYMV.

The main objective of this study is to focus on the ABA-virus interaction with an emphasis on the effect of ABA inhibition on viral infection process and viral titer. The study is all about measuring the effect of ABA inhibitor of the viral titer through measuring some physiological and biochemical parameters to compare between the healthy, infected and ABA inhibited and BYMV infected Lupine plants.

## Materials and Methods

### Chemicals and reagents

The following reagents were from Sigma Chemical Co., USA: bovin serum albumin; Abscisic acid inhibitor NDGA, and ABA Phyto Detection Kit. Monoclonal antibodies for BYMV from Agdia company, USA. RT-PCR kit has been brought from Invitrogen, USA.

### Virus isolation and identification

BYMV inoculum was obtained from infected *Vicia faba* leaves showing mosaic and malformation were chosen for mechanical inoculation of diagnostic host plant, *Chenopodium Amaranticolor* and healthy lupine seedlings. Both plants were kept in an insect free greenhouse. Selected leaves were ground using a pestle and mortar with a little acid washed sand and distilled water (1:2 w/v). The bulk of the leaf debris and sand was removed by squeezing the pulp through three layers of muslin. The extract was centrifuged at 4,000 x g for 15 min, and the supernatant decanted and kept at room temperature overnight to precipitate any proteinaceous virus inhibitor presented in the leaf sap. The supernatant was clarified by further centrifugation at 3,000 x g for 15 min. The inoculated plants showing infection symptoms were used later for inoculations. The prepared virus inoculum was used in two ways: one to inoculate *Chenopodium Amaranticolor* as differential host showing local lesions as follows: leaves were dusted and mechanically inoculated with BYMV and total number of local lesions of 10 leaves were calculated after 7 days of inoculation; and second, to inoculate the seedlings of lupine. Lupine plants developed symptoms after three weeks of inoculation. Greenhouse was used to keep inoculated plants under observation.

### Detection of BYMV coat protein gene by RT-PCR

A confirmation of the identity of the virus was achieved by amplifying the coat protein (CP) gene of the test virus. Total RNA was isolated from 100 mg of infected plant leaves using Trizol (Invitrogen, USA) as per the manufacturer instruction. The integrity of total RNA was analyzed on 1% denaturing gel using leaves from healthy plants as negative control. cDNA was synthesized using reverse transcriptase in a reaction volume of 20 µl, containing 1 µg of total RNA, 1 µl of oligodT (10–18) as per the manufacturer's instructions. For reverse transcription-polymerase chain reaction (RT-PCR), BYMV specific primer pair BYMV-pnsF (5`-TCAGATCAAGAGCAACTCAATGCA-3`) and BYMV-pnsR (5`-GACGGATACTCTAAATACGAACA-3`) [17] specifically designed from the BYMV coat protein gene. RT-PCR amplification was performed in 50 µl reaction volume using 5 µl of 10 x Taq buffer (Invitrogen), 3 µl of 25 mM $MgCl_2$, 4 µl of 2 mM dNTPs mix, 2 µl of 10 mM forward and reverse primer, 2.0 µl of cDNA, 0.2 µl of 5 U/µl Taq polymerase and final volume was adjusted with nuclease free water.

Amplification was carried out in thermal cycler programmed as follows: 95°C/15 min (1 cycle); 95°C/1 min; 45°C/1 min; 72°C/2 min (40 cycles); 72°C/5 min (1 cycle); 4°C (infinitive). Agarose gels (1.2%), used for resolving the PCR products, were prepared and run as described by Maniatis et al. The gel was stained with ethidium bromide and evaluated under UV light. The sizes of PCR products were estimated according to the migration pattern of a 100 bp DNA ladder [18].

### Plant treatment

Lupine leaves were treated with ABA biosynthesis inhibitor NDGA, which targets the synthesis of 9-cis-epoxycarotenoid dioxygenase, which is the key enzyme of ABA. Three concentrations of NDGA were prepared: 5, 15, and 30 mM. ABA was prepared at 100 µM and all solutions were dissolved in 0.2% Ethanol. Mock controls were treated with 0.2% Ethanol alone. Solutions were sprayed on both sides of leaves until they were completely coated. After 12 h, leaves were mechanically infected with BYMV virus; these plants were kept under greenhouse conditions for three weeks until the mosaic symptoms appear. Previous studies reported that low concentrations of NDGA (<1 mM) show very weak inhibition for the ABA, and higher concentrations have more effect on the ABA [19], concentration 30 mM of ABA inhibitor will be used in all experiments studying the influence of ABA inhibitor on the BYMV infection and on the physiological parameters of Lupine.

### Effect of BYMV infection on different physiological parameters of lupine

Pigment contents, water soluble carbohydrates, total proteins, total free amino acids, proline content, total phenolics, SA, and concentration of ABA were determined in the leaves of the lupine plants at different time intervals, 1, 2, and 3 weeks from inoculation with BYMV. Lupine plants were grown in 10 cm diameter pots under natural conditions in a greenhouse at 25°C. Seedlings of 4-5 leaf stage were selected for different analysis. Mechanical inoculation was done by using a sap prepared from virus infected *C. Amaranticolor* leaves ground in phosphate buffer, pH 7.0 using carborundum (600 mesh) as an abrasive to inoculate. Control plants were treated only by buffer in presence of dusted carborundum. Three replicates of the plant were used per period for each test. Leaves collected were at the same stage. Three weeks after inoculation the youngest fully developed leaves from both control and treated plants were collected for analysis of biochemical changes.

All the test parameters will be measured in healthy Lupine plants, BYMV infected plants, and ABA inhibitor treated and BYMV infected plants. Details about each tested parameter are shown below.

**Quantitative determination of chlorophylls:** Leaves from inoculated, treated and control Lupine plant were rapidly frozen in liquid nitrogen, ground to a powder and the chlorophyll was extracted

with 80% v/v acetone, total chlorophyll content was measured by Porra et al., [20].

**Estimation of total carbohydrate content:** Plant extract (100 mL) was taken in 25 mL test tubes and 6 mL anthrone reagent (150 mg of another one in 72% $H_2SO_4$.) was added, and then heated in boiling water bath for 10 min. The test tubes were ice cooled for 10 min and incubated for 25 min at 25°C. Optical density (OD) was read at 625 nm on a spectrophotometer. The carbohydrate content was calculated from the standard curve using glucose with the same method which mentioned above.

**Estimation of total protein content:** Total protein was estimated calorimetrically according to Bradford method [21] by recording absorbance at 595 nm. Bovine serum albumin was used as standard. Protein content in leaf samples was recorded as μg of protein per g of leaf.

**Determination of the total soluble protein:** 0.5 g of leaves of healthy and infected plants was ground in a volume of 0.1 ml (SDS) sample buffer solution. Extracts were added in 1.5 cm eppendorf centrifuge tube according to Laemmli (1970). Homogenates were heated at 95°C for 5 min then briefly centrifuged at 12,000 rpm to pellet cellular debris. The resulting supernatants (total protein extracts) were stored at -70°C until analysis by PAGE. The extract was separated by electrophoresis on 1 mm thick 12.5% acrylamide slab gels. Gels were stained with Coomassie blue.

**Amino acids analysis**: Free amino acids content mg/g dry weight was determined colorimetrically according to the method described by Jayarman [22].

**Estimation of proline content:** Proline content of lupine leaves was determined according to Bates et al., [23]. A known dry weight (0.1 g) of leaves was extracted in 10 ml of aqueous 3% sulfosalicylic acid overnight. The extract was centrifuged at 1500 rpm for 10 mins. A total of 2 ml of the supernatant were mixed with 2 ml of fresh acid ninhydrin solution for reaction and 2 ml glacial acetic acid in a test tube for 1 h at 100°C. The reaction was terminated in an ice bath, and the mixture was extracted with 4 ml toluene. The extract was vigorously stirred for 20s using a test tube stirrer. Therefore, the chomophore-containing toluene was separated from the aqueous phase, and its absorbance was measured at 520 nm. The proline content was determined from a standard curve.

**Estimation of total phenolics content:** The phenol content was estimated using Folin-Ciocalteau reagent. Ethanol (80%) was used for extraction of phenols. Five g plant material were ground in two 25 ml of 80% ethanol and centrifuged. The extracts were pooled and made up to 10 ml. Ethanol extract (0.5 ml) was evaporated on a water bath, to which 6 ml water was added and shaken well before addition of 0.5 ml Folin-Ciacalteau reagent. After 5 min, 2 ml of 20% sodium carbonate solution was added. After incubation for 30 min, absorbance at 660 nm was measured. Using pyrocatechol as standard, the phenol content in the leaf extract was calculated [24].

**Determination of the SA concentrations:** Samples were dissolved in the solvent followed by centrifugation at 10,000 g for 10 min. The supernatant was stored on ice for SA measurement. 100 μl of the supernatant was mixed with 0.1% freshly prepared ferric chloride. The volume of the reaction mixture was made up to 3.0 ml and the complex formed between $Fe^{3+}$ ion and SA, which is violet in color was determined by spectrophotometry, measuring the absorbance of the complex in the visible region (at 540 nm). SA measurements were carried out with different lupine samples and the amount of SA in the leaf samples was determined accordingly [25].

**Determination of abscisic acid concentration by Enzyme-linked Immunosorbent Assay (ELISA):** Abscisic acid (ABA) content from leaves of healthy, infected and ABA treated and infected Lupine, was determined using the Phytodetek ABA kit (Sigma, St Louis, MO, USA). Ten milligrams of leaves were homogenised in 500 μL of cold TBS buffer (Tris-HCl NaCl) and centrifuged for 10 min at 14000 g. One hundred microlitres of supernatant was added to ELISA wells coated with antibodies to ABA. The ABA concentration was determined as described by the manufacturer.

### Determining the accumulation of BYMV in healthy, infected and ABA inhibitor treated and infected with BYMV by ELISA

The virus was detected by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). The BYMV-specific ELISA kit was obtained from the Agdia Company (Elkhart, USA). Leaves of infected, healthy, and ABA inhibitor treated and infected lupine plants were homogenized in phosphate buffered saline (PBS; 10 mM $Na_2HPO_4$ , 0.14 M NaCl, pH=7.0) at 1 g: 10 ml. The extracts were filtered through double layers of muslin and tested by DAS-ELISA as described by Clark and Adams. Absorbance values that were twice the value of healthy plants at 405 nm were considered positive.

## Results and Discussion

### Detection of BYMV coat protein gene by RT-PCR

In order to identify the BYMV, in the infected plants showing the mosaic symptoms, the total RNA from the leaves of the infected plants was extracted and used to amplify the coat protein gene specific for BYMV by using specific primers by reverse transcriptase polymerase chain reaction which revealed an amplified product of molecular size 800 bp characteristic for the coat protein gene (Figure 1). Similar results were obtained by Kaur et al., [26].

### BYMV infection, symptoms expressed in the infected plants

In order to identify the BYMV infection in diagnostic host plant species, *Chenopodium Amaranticolor* was used as a characteristic local lesion host. Necrotic lesions appeared 7 days after mechanical inoculation followed by deformation of the upper leaves 14 days after inoculation. These symptoms were typical for the infection of BYMV on the host plant.

The BYMV infected Lupine plants showed narrower leaflets, severe mosaic and leaf deformation, with percent of infection incidence of 85%. A successful propagation of the BYMV infection has been done in Lupine plants under greenhouse conditions. Similar work has been reported by Piche and Peterson [27] who isolated BYMV from white Lupine. The results obtained previously from the symptoms appeared on the indicator plant and the results from the molecular studies provide evidence that the virus causing mosaic, distortion of the younger leaves and yellowing on Lupine plants is an isolate of BYMV.

### Plant treatment

Lupine leaves were treated with three concentrations of ABA inhibitor, NDGA: 5, 15, and 30 mM, and after 12 h, leaves were mechanically infected with BYMV virus.

Results in (Table 1) below show that usage of ABA inhibitor compounds decreased the percentage of BYMV infection considerably using ABA inhibitor treatment. This decrease was inversely proportional

to the amount of ABA inhibitor used, as the inhibitor amount increases the virus infection kept decreasing, at 5 mM, 15 mM, and 30 mM of ABA inhibitor treatment. This comes in accordance with Mazen et al., [28], who found out that low concentrations of ABA inhibitor do not affect the viral infection like the bigger concentrations.

Abscisic acid has been considered a negative regulator of disease resistance [10]. This negative effect appears to be due to the interference of abscisic acid with biotic stress signaling that is regulated by salicylic acid, jasmonic acid and ethylene and to an additional effect of ABA on shared components of stress signaling. ABA will negate the effect of the defense controlled by other pathways signaling like SA, and JA [29].

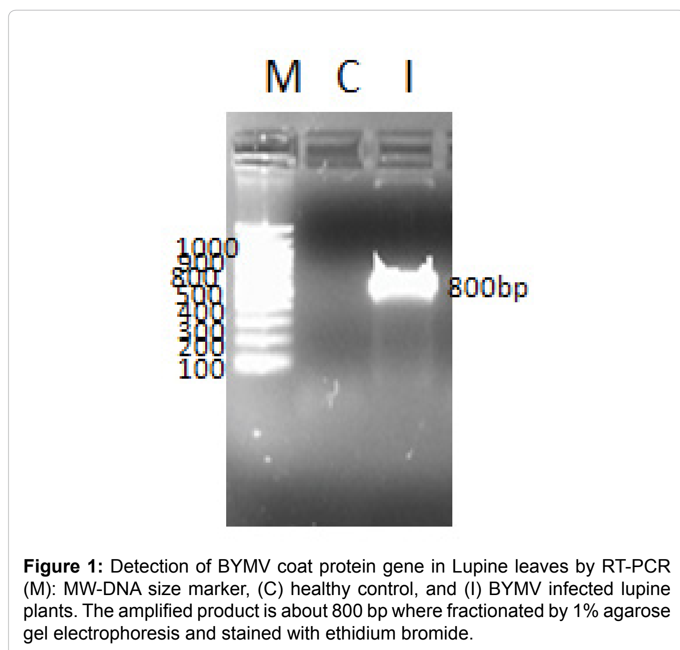


**Figure 1:** Detection of BYMV coat protein gene in Lupine leaves by RT-PCR (M): MW-DNA size marker, (C) healthy control, and (I) BYMV infected lupine plants. The amplified product is about 800 bp where fractionated by 1% agarose gel electrophoresis and stained with ethidium bromide.

| Weeks after inoculation | % of Infection | | % of Infection | % of Infection |
|---|---|---|---|---|
| | Infected | 5mM ABA Inhibitor + virus | 15mM ABA Inhibitor + virus | 30mM ABA Inhibitor + virus |
| 3 | 85 | 72.7 | 65.9 | 50.15 |

**Table 1:** The effect of spraying different concentrations of ABA inhibitor on the % of infection of BYMV on Lupine.

## Effect of BYMV infection on different physiological parameters of lupine quantitative determination of chlorophylls

Lupine plants infected with BYMV showed a gradual decline in the photosynthetic pigment. This reduction was observed in chlorophyll a and chlorophyll b throughout the infection course. The concentration of the pigment after the first, second, and third week of inoculation achieved 75.1% 50.8%, and 27.0% of their corresponding healthy control content respectively. Chlorophyll b showed also a decline in the BYMV infected Lupine leaves over the healthy control plants. Chlorophyll b content values achieved 86.1%, 55.0%, and 19.0% of their corresponding values at the healthy Lupine plants after first, second, and third week of infection respectively. Virus infection usually affects the process of photosynthesis. Reduction in carbon fixation is the most commonly reported effect in leaves showing mosaic diseases. This reduction usually becomes detectable some days after infection. Photosynthetic activity can be reduced by changes in chloroplast structure, by reduced content of photosynthetic pigments [30].

ABA inhibitor treated and BYMV infected Lupine plants also showed a gradual decline in the concentration of chlorophyll a and b values than the control healthy plants. The ABA inhibitor treated and infected plants showed however a considerable increase in the chlorophyll a and b content values over the infected ones. Recorded values were 82.7%, 68.3%, and 51.1% of the healthy chlorophyll a content after the first, second, and third weeks of infection respectively, and 90.2%, 63.8%, and 39.4% of the healthy plant chlorophyll b value after the first, second, and third weeks respectively. Spraying with ABA inhibitor helps the plant get rid of the increasing level of ABA due to infection, and induce more resistance to the stress and consequently have a positive effect on the chlorophyll concentration.

Comparable results were reported by DongDong et al., [31], who investigated the effect of exogenous ABA and ABA inhibitor (NDGA) on the ripening of strawberry, and found out that chlorophyll content was higher in the ABA inhibitor treated fruit than the healthy control (Figure 2A and 2B). Shahrukh et al., [32], on the other hand studied the morpho-physiological parameters of the banana upon an infection with the banana bunchy top virus (BBTV), and found out that the chlorophyll content decreased significantly in the infected plant comparing with the healthy control (Table 2).

**Estimation of total carbohydrate conten:** Results in Table 3 and

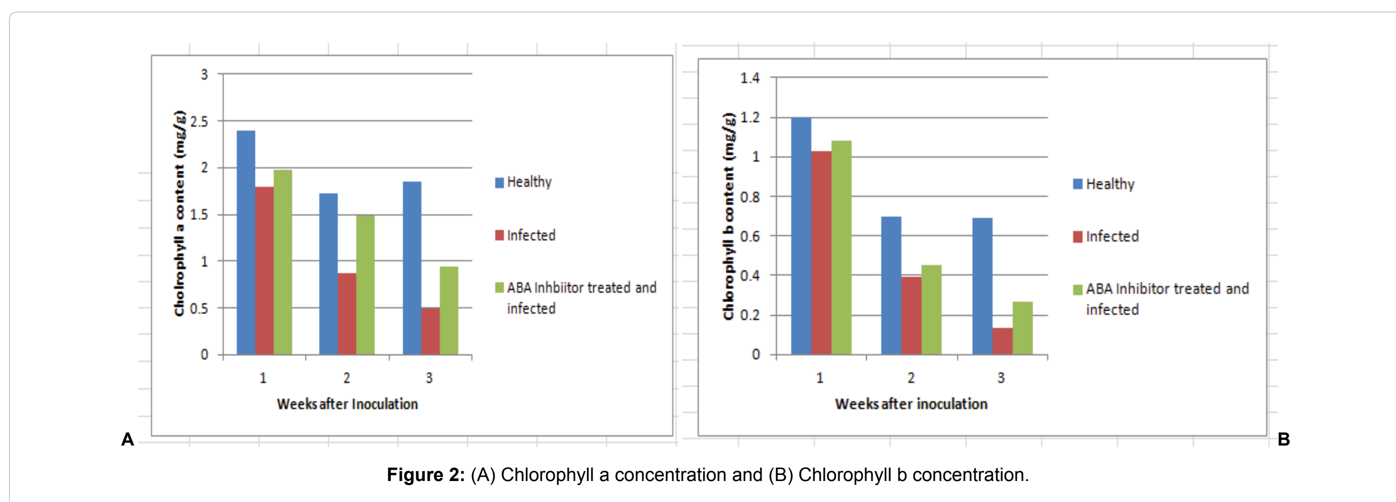


**Figure 2:** (A) Chlorophyll a concentration and (B) Chlorophyll b concentration.

| Weeks after inoculation | Chlorophyll a content (mg/g) | | | | | Chlorophyll b content (mg/g) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | ABA Inhibitor treated and Infected | % of relative content | Healthy | Infected | % of relative content | ABA Inhibitor treated and Infected | % of relative content |
| 1 | 2.4 | 1.80 | **75.1** | 1.98 | **82.7** | 1.2 | 1.03 | **86.1** | 1.08 | **90.2** |
| 2 | 1.73 | 0.878 | **50.8** | 1.49 | **68.3** | 0.7 | 0.39 | **55.0** | 0.45 | **63.8** |
| 3 | 1.85 | 0.499 | **27.0** | 0.94 | **51.1** | 0.689 | 0.134 | **19.0** | 0.27 | **39.4** |
| % of relative content is the content in infected or treated over the control *100 ;If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease | | | | | | | | | | |

**Table 2:** Chlorophyll a and chlorophyll b contents of the healthy control, BYMV infected and ABA inhibitor treated and infected lupine.

| Weeks after inoculation | Total carbohydrates content in (mg/g) | | | | |
|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | ABA inhibitor treated and Infected | % of relative content |
| 1 | 10.2 | 6.4 | **62.6** | 8.1 | **79.6** |
| 2 | 23 | 8.6 | **37.3** | 12.8 | **55.8** |
| 3 | 25 | 9.1 | **36.4** | 14.3 | **57.2** |
| % of relative content is the content in infected or treated over the control *100; If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease | | | | | |

**Table 3:** Total carbohydrates in (mg/g) for different treatments of Lupine plants as time changes.

Figure 3 below show that, total carbohydrate content of infected plants was lower than expected when compared to healthy control. Values started at 62.6% of the healthy Lupine plants at the first week after inoculation, reached 37.3% of the healthy corresponding value at the second week, and finally reached 36.4% at the third week of inoculation. This result may be due to the increase in respiration, which will make the plant consume more carbohydrates than usual, or the decrease in photosynthetic pigment which in turn decreases $Co_2$ fixation and photosynthetic process reflecting lower amount of carbohydrates. These results come in agreement with these reported by Manisha et al., [30], who studied the effect of infection of Dolichos lablab plant with the bean common virus and found out that the respiration rate in the infected plant leaves was always higher than its corresponding value in the healthy control leaves, which made the carbohydrate content lower in the infected plant leaves than its corresponding value in the healthy control leaves.

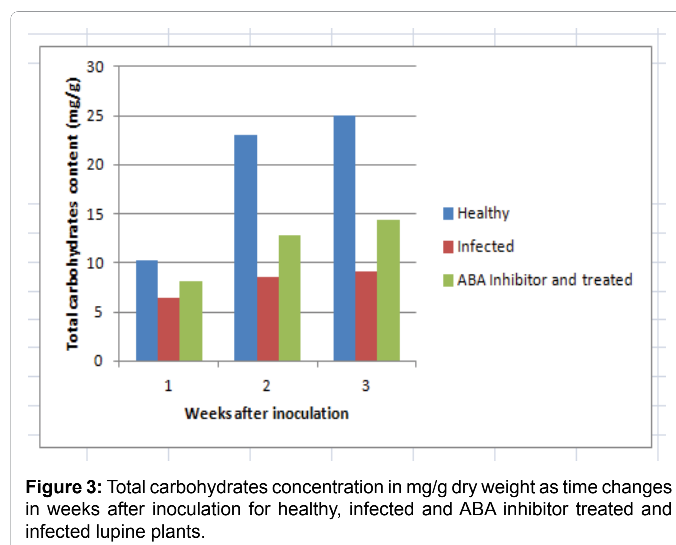


**Figure 3:** Total carbohydrates concentration in mg/g dry weight as time changes in weeks after inoculation for healthy, infected and ABA inhibitor treated and infected lupine plants.

The ABA inhibitor treated and infected lupine plants showed a decrease also in the level of carbohydrates concentration comparing to the control healthy plants. The carbohydrates' contents however in the ABA inhibitor treated and infected was higher than their corresponding values in the infected plants. Results recorded 79.6%, 55.8%, and 57.2% of carbohydrates content in the ABA treated and infected Lupine plants after first, second, and third weeks of infection respectively. This may be due to the fact that inhibiting the ABA in the Lupine plants increases the level of chlorophyll in the plant which in turn keeps the photosynthetic pigments and consequently the carbohydrates content very close to the healthy control values even though the virus titer may be as high as the infected plants. Results also showed the increase of the level of carbohydrates as the Lupine ages.

**Estimation of total protein content:** The protein concentration decreased in BYMV infected Lupine plants compared to the healthy control plants. Results in infected plants recorded 93.7%, 92%, and 80% of protein content comparing to its corresponding values in the healthy control plants after one, two, and three weeks of infection respectively. Comparable results were reported by Chatterjee et al., [33], who studied the changes in biochemical parameters of mesta plants upon infection with the monopartite Begomo virus and reported the decline in total protein in the infected plants over than the corresponding value in the healthy plants. Opposing results on the other hand, were reported by Manisha et al., [30] who found out an increase in the total protein content of the bean common virus infected Dolichos lablab plants over the healthy ones. This distinction in the results may be due to the variation in the virus-host combinations. ABA inhibitor treated and infected lupine plants on the other hand showed an increase in its protein content comparing to the infected plants. Results showed 121%, 115%, and 104% of protein content in ABA treated and infected plants comparing to the health control values after the first, second, and third weeks of infection respectively, as shown in Table 4 and Figure 4 below, which may be due to the release of any defense related proteins that the abscisic acid was blocking. These results support also the defense role of proteins as secondary metabolites [34].

**Determination of the total soluble protein:** Infected lupine plants showed variable changes in band patterns compared with control healthy ones. The virus infection caused some new protein bands to show up at 22, 27, 35, and 39 kDa in infected lupine plants and they did not exist in the healthy ones. Indicating that viral pathogen induced the synthesis of pathogenesis related proteins in inoculated plants [34]. As for the ABA inhibitor and BYMV infected lupine plants, there were five bands, two of them (35, and 39 kDa) persisted from the infected plants, and three new ones showed up at 24, 65, and 70 kDa, which may be due

| Weeks after inoculation | Protein content (mg/g) | | | | |
|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | ABA Inhibitor treated and Infected | % of relative content |
| 1 | 0.51 | 0.47 | **93.7** | 0.61 | **121** |
| 2 | 0.64 | 0.58 | **92.0** | 0.73 | **115** |
| 3 | 0.76 | 0.60 | **80.0** | 0.79 | **104** |
| % of relative content is the content in infected or treated over the control *100; If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease. | | | | | |

**Table 4:** Protein content of healthy, BYMV infected and ABA inhibitor treated and infected lupine.

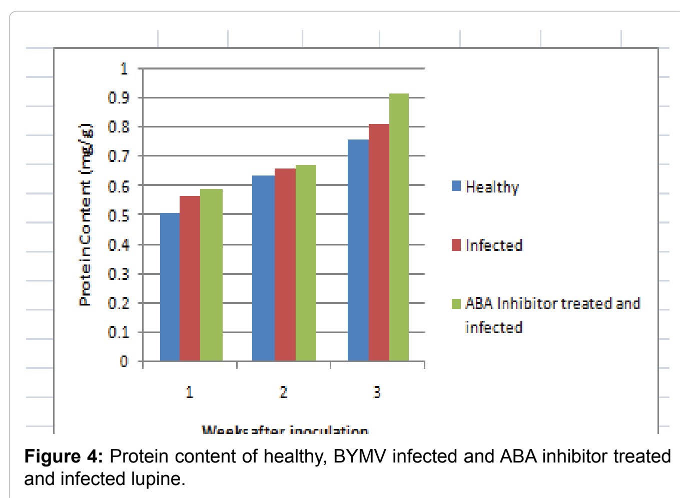


**Figure 4:** Protein content of healthy, BYMV infected and ABA inhibitor treated and infected lupine.

to the effect of suppression of ABA biosynthesis as a result of the ABA inhibitor treatment allowing the appearance of new proteins to resist the virus. The result of the study indicated that BYMV infection, and ABA devoid and infected plants significantly changed the biochemical expression level of protein pathway. These results come in agreement with the results reported by Liu et al., [35] who found discovered an identification of 38 proteins expression in cucumber in response to an infection with cucumber green mottle virus.

Also, the protein pattern profile of both infected and ABA inhibitor treated and infected Lupine plants showed protein bands of molecular weight 35 kDa characteristic for the BYMV coat protein, this kind of protein was totally absent in the profile of healthy Lupine plants (Figure 5). These results come in accordance with [36].

**Amino acids analysis:** BYMV Infected Lupine leaves showed greater values of free amino acid concentration than the control lupine plants after one week of inoculation and this increase in the amino acid concentration persisted till the third weeks after inoculation. Infected plants showed a relative content of 130%, 125%, and 127% of total free amino acid content in the first, second and third week of infection respectively, as shown in Table 5 and Figure 6. This increase in the free amino acid content together with the decline in the total protein, confirms the possibility of the virus enhancing the breakdown of the proteins and polypeptides to have a reservoir of amino acids for viral biosynthesis, and consequently increased the total free amino acid contents. Comparable results were reported by Chatterjee and Ghosh [33].

ABA inhibitor treated and infected plants on the other hand, showed higher values of total free amino acid content comparing to the control healthy plants, but lower than the corresponding infected ones.

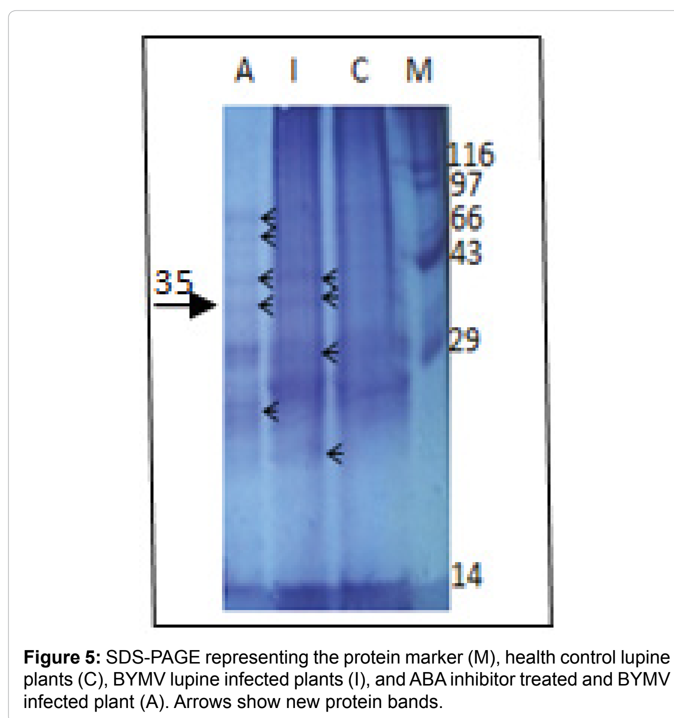


**Figure 5:** SDS-PAGE representing the protein marker (M), health control lupine plants (C), BYMV lupine infected plants (I), and ABA inhibitor treated and BYMV infected plant (A). Arrows show new protein bands.

| Weeks after inoculation | Total free amino acid (mg/g) | | | | |
|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | ABA Inhibitor treated and Infected | % of relative content |
| 1 | 200 | 260.6 | **130** | 224 | **112** |
| 2 | 205.4 | 256.75 | **125** | 211.5 | **103** |
| 3 | 192 | 245 | **127** | 205.4 | **107** |
| % of relative content is the content in infected or treated over the control *100; If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease. | | | | | |

**Table 5:** Total free amino acid for healthy, BYMV infected and ABA treated and infected Lupine.

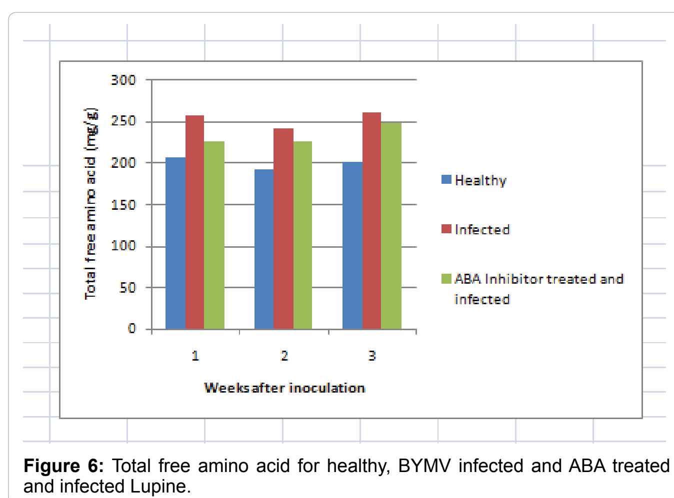


**Figure 6:** Total free amino acid for healthy, BYMV infected and ABA treated and infected Lupine.

Results recorded 112%, 103%, and 107% relative content after the first, second, and third weeks of infection respectively.

**Estimation of proline content:** Role of proline metabolism is

tightly regulated in plants especially during pathogen infection and its role has not yet been fully understood [37].

Proline content in BYMV infected lupine plants was observed to be much higher than its corresponding values in the healthy control plants. Relative content of proline in infected plants reached 320%, 330%, and 289% comparing to the healthy control, after first, second, and third weeks of infection respectively, as shown in Table 6 and Figure 7 below. Comparable results were obtained by Pazarlar et al., [38] who studied the proline content of the pepper plants as a result of infection with tobacco mosaic virus infection and found out the increase in the level of the proline content in the infected plant over the healthy control.

Results also showed that the ABA inhibitor treated and BYMV inoculated lupine plants showed higher levels of proline content than the healthy control but less than the corresponding infected plants. Values reached 117%, 166%, and 167% of the content in the healthy plants after the first, second, and third weeks of infection respectively. These results can be explained by the fact that ABA encourages proline accumulation. Similar results were reported by Lobato et al., [39], who found out that water deficient stressed plants contain more proline content than the healthy ones due to the ABA increase as a result of the stress. Proline is an important component of structural protein in both animals and plants as a protecting protein against abiotic and biotic stress [40]. When Lupine was exposed to virus infection, it produced reactive oxygen species (ROS) that induced programmed cell death in the plant tissues surrounding the infection. Proline acts as a scavenner of ROS and prevents ROS production of programmed cell death [33].

**Estimation of total phenolics content:** Total phenolics concentration showed a big increase in BYMV infected lupine plants. Values recorded were 302%, 377%, and 254% comparing to the corresponding healthy control values after the first, second, and third weeks of infection correspondingly. Comparable results were reported by Alawlaqi et al., [41] who reported the increase in phenolics concentration in the zucchini yellow mosaic virus infected cucurbit plants comparing to the healthy plants.

Concentration of phenolics in ABA inhibitor treated and infected plants showed also an increase in the phenolics content with respect to the control and infected plants for all tested weeks after inoculation. Results showed a phenolics content of 413%, 420%, and 300% relative to the healthy control content after the first, second, and third weeks of infection respectively (Table 7 and Figure 8 below), confirming the defense role of these metabolites. Phenolics have been found to have several functions. They function in lignin biosynthesis, regulation of plant responses to abiotic stimuli, function in pigmentation, growth, reproduction, resistance to pathogens, and many other functions [42].

**Determination of the SA concentrations:** Salicylic acid content showed a slight increase in lupine plants infected with the BYMV. Infected plants achieved 120%, 114%, and 108% of the SA content in corresponding healthy plants, which can be due to the induction of SA upon infection. ABA role as a plant hormone regulating seed germination and fruit ripening [31] is well known, as well as its antagonistic role in defense pathways of SA, JA, and ET [43]. ABA inhibitor treated and infected lupine plants, on the other hand had a

| Weeks after inoculation | | | Proline content (mg/g) | | |
|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | ABA Inhibitor treated and Infected | % of relative content |
| 1 | 1.7 | 5.44 | **320** | 2.9 | **117** |
| 2 | 2.1 | 6.95 | **330** | 3.5 | **166** |
| 3 | 2.8 | 8.1 | **289** | 4.7 | **167** |
| % of relative content is the content in infected or treated over the control *100; If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease. | | | | | |

**Table 6:** Proline content of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

| Weeks after inoculation | Phenolics Concentration (mg/g) | | | | |
|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | ABA Inhibitor treated and Infected | % of relative content |
| 1 | 139.7 | 421.9 | **302** | 577.5 | **413** |
| 2 | 145.3 | 548.3 | **377** | 611.2 | **420** |
| 3 | 291.4 | 741 | **254** | 874.2 | **300** |
| % of relative content is the content in infected or treated over the control *100; If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease. | | | | | |

**Table 7:** Phenolics concentration of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

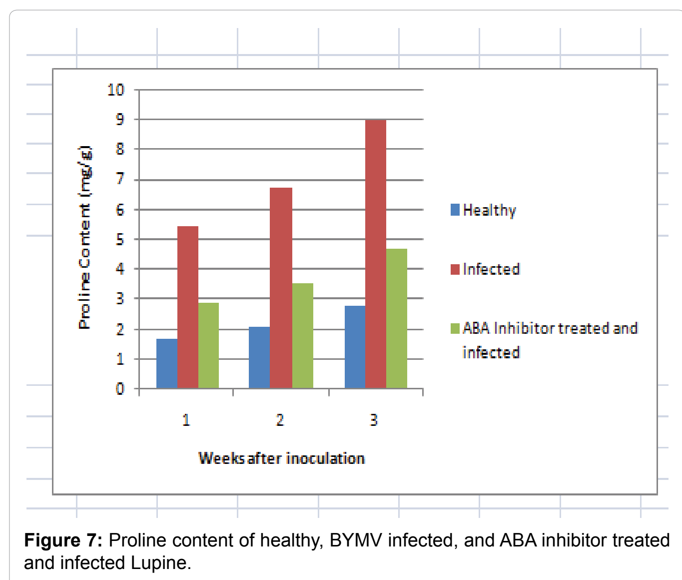


**Figure 7:** Proline content of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

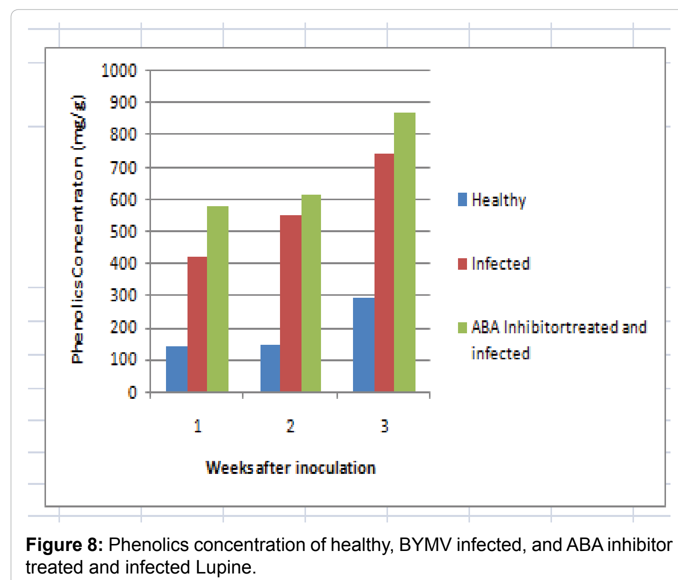


**Figure 8:** Phenolics concentration of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

bigger increase in the SA content comparing to the healthy control, and the infected plants. Values achieved 149%, 168%, and 172% of the SA content comparing to healthy control plants after the first, second, and third weeks of infection, which can be due to inhibition of ABA, which in turn will lead to more SA induction. This increase gets even bigger with time after 3 weeks from inoculation, as shown in Table 8 and Figure 9 below. This increase in SA affects the viral disease development. The lower SA gets the lower the resistance of the plant toward virus infection.

**Determination of abscisic acid concentration**: Little is known about the primary causes of ABA-induced resistance. Results showed a sharp rise in the level of endogenous ABA of lupine plants happened at an early stage of BYMV infection values. ABA content 346% of relative content in the infected plant comparing to the healthy control Lupine plants. The ABA content of BYMV infected Lupine plants increased more in the second week of BYMV infection reaching 383% of its corresponding value in the control health plant.

The increase reached a peak at the third week of virus inoculation reaching 500% its corresponding ABA content value in the healthy control plant (Table 9 and Figure 10 below).

On the other hand, Lupine plants treated with ABA inhibitor and infected with BYMV resulted in a very lower level of ABA compared with healthy control, as indicated by ABA biosynthesis inhibition.

Using different concentrations of NDGA to inhibit the ABA content affected the content of ABA dramatically declining to 37.7% after the first week of infection using the 5 mM ABA inhibitor and reaching 25.5% after the third week of infection. Using more concentrations of the ABA inhibitor caused more decline in the ABA content in the lupine plants comparing with the healthy control plants. Presence of ABA in certain concentration inside the plant is important for the development of the disease susceptibility in Arabidopsis exposed to fungal infection [10]. To determine whether the reduction in viral titer was a consequence of its decreased ABA content in ABA inhibitor treated and infected plants the next experiment was conducted to measure the virus accumulation (Table 9).

## Enzyme-linked Immunosorbent Assay (ELISA) for determining the accumulation of BYMV in healthy, infected and ABA inhibitor treated and infected with BYMV

Double antibody sandwich-enzyme linked immuno-sorbent assay (DAS-ELISA) was used to determine the concentration of the BYMV in infected and ABA inhibitor treated and infected Lupine plants.

Results are shown below in (Table 10) where +ve control is 1.67

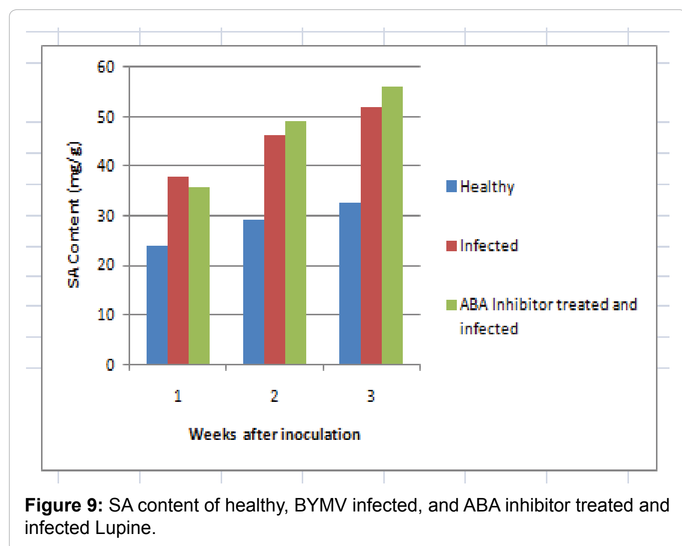


**Figure 9:** SA content of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

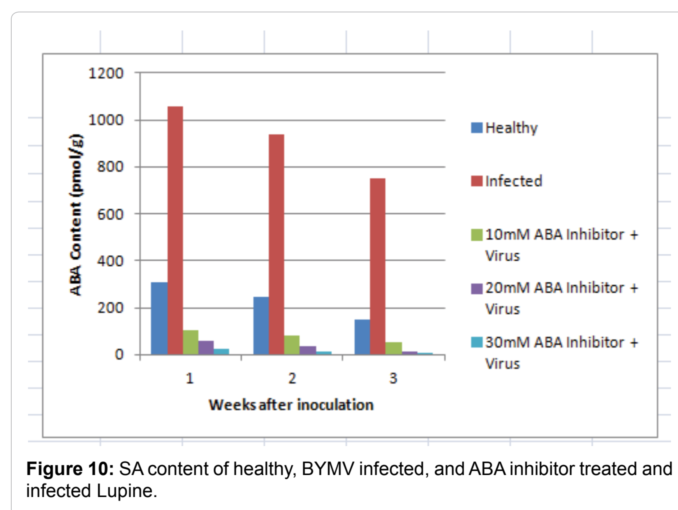


**Figure 10:** SA content of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

| Weeks after inoculation | SA Content (mg/g) | | | | |
|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | ABA Inhibitor treated and Infected | % of relative content |
| 1 | 23.9 | 28.8 | **120** | 35.8 | **149** |
| 2 | 29.1 | 33.4 | **114** | 49.1 | **168** |
| 3 | 32.5 | 35.1 | **108** | 56.1 | **172** |
| % of relative content is the content in infected or treated over the control *100; If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease. | | | | | |

**Table 8:** SA content of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

| Weeks after inoculation | ABA Content (pmol/g) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Healthy | Infected | % of relative content | 5mM ABA Inhibitor + virus | % of relative content | 15mM ABA Inhibitor + Virus | % of relative content | 30mM ABA Inhibitor + virus | % of relative content |
| 1 | 305.6 | 1059.2 | **346** | 115.4 | **37.7** | 57.3 | **18.7** | 26.1 | **8** |
| 2 | 243.4 | 935.9 | **384** | 70.5 | **29.0** | 27.5 | **11.3** | 15.0 | **6** |
| 3 | 150.3 | 752.1 | **500** | 38.4 | **25.5** | 9.76 | **6.5** | 3.11 | **2** |
| % of relative content is the content in infected or treated over the control *100; If % of relative content >100 then the increase above the 100 is the actual increase; If % of relative content <100 then the decrease from the 100 is the actual decrease. | | | | | | | | | |

**Table 9:** ABA content of healthy, BYMV infected, and ABA inhibitor treated and infected Lupine.

| Treatments | Weeks after inoculation | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | |
| | Virus Conc. | Infection % | Virus Conc. | Infection % | Virus Conc. | Infection % |
| Healthy | 0.031 | | 0.083 | | 0.150 | |
| Infected | 1.028 | **60.4** | 1.381 | **78.7** | 1.665 | **91.4** |
| 5 mM ABA Inhibitor+virus | 0.886 | **52.08** | 1.275 | **72.2** | 1.653 | **90.8** |
| 15 mM ABA Inhibitor+virus | 0.714 | **41.9** | 0.992 | **56.5** | 1.543 | **84.7** |
| 30 mM ABA Inhibitor+virus | 0.582 | **34.2** | 0.816 | **46.5** | 1.278 | **70.2** |
| ELISA readings were recorded after 30 mins incubation with the pnpp substrate at 450 nm wave length. ELISA readings greater than twice absorbance values of the healthy control was considered +ve. OD data recorded as mean of optical absorbance. | | | | | | |

**Table 10:** Mean ELISA values OD (405) of duplicate sample for extracts of leaves of lupine infected with BYMV.

and healthy lupine plants will serve as the –ve control. Results show that Virus infection increased gradually for week 1 after inoculation until reached a peak of 91.4% of infection on the third week in infected Lupine plants. These results show that elevated level of ABA infected plants play a role in increasing the infection, indicating the major role ABA plays in the susceptibility of lupine to BYMV. Comparable results were reported by Ulferts (2015) who confirmed the negative role of ABA when applied exogenously on barley against the plant fungus *Magnaporthe oryzae*, and increased the disease susceptibility.

Applying the different concentrations of the ABA inhibitor on the Lupine leaves and after 12 hours inoculated with the BYMV, on the other hand, decreased the virus infection considerably, and as the concentration of the ABA inhibitor increases the virus infection declines reaching a value of 70.2% on the third week of virus infection after treating the Lupine leaves with 30 mM ABA inhibitor before inoculation.

From the previous results, it can be concluded that using ABA inhibitor can influence the viral infection process in the form of virus concentration, percentage of infection and severity of disease comparing with the BYMV infected plants. Lupine plants with inhibited or reduced level of ABA were much more resistant to BYMV as indicated in the level of virus accumulation suggesting its possible use as a plant regulator or chemotherapeutic agent for plant virus infection. Also, activation of ABA signals during infection processes decreases the accumulation of defense compounds that are associated with virus infection. An important point to note here is that infection declined and reached 50.15% of its original value (85%) when spraying 30 mM ABA inhibitor on the Lupine plant and check for the symptoms. The infection reached however, only 70.2% of its original value (91.4%) when calculated from virus concentration using ELISA. This means that using the ABA inhibitor reduced the virus symptoms to a certain degree. However, the actual virus titer was higher than what the symptoms suggest when using the ELISA to measure the virus accumulation. This indicates that ABA biosynthesis inhibition enhances physiological and biochemical repair in both primary and secondary metabolites with little impact on the viral titer.

## Limitations of the Study and Future Research

This study did not investigate the relation between the infection and other growth regulator substances like ethylene, and jasmonic acid. Future research is needed to elucidate the generation mechanism and relationship between virus pathogenesis and oxidative stress and their role in the symptoms development.

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