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Effect of Using *Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina platansis* Alga on Bean Yellow Mosaic Virus (BYMV) Infection in Two Varieties of Broad Bean

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ABSTRACT

The study aimed at evaluating the efficiency of *Bacillus subtilis* bacterium, *Ganoderma lucidum* fungus, and *Spirulina platansis* alga extract on two varieties (Barcelona and a local variety) of Broad bean plants in an attempt to reduce the negative impact of yellow mosaic virus on broud bean (BYMV) through stimulating plant systemic resistance. The results obtained showed significant effects of the biocontrol agents investigated in reducing the severity of infection and increasing growth parameters of the treated plants in both individual and combinational treatments. The triple combinational treatment with Barcelona variety achieved a significant difference in reducing the severity of infection by 16.17% as compared with that of the corresponding treatment with the local variety. Additionally, a significant increase in growth parameters including, plant height, leaf area, chlorophyll ratio, and fruit setting ratio of 42.33 cm, 44.77 cm^2, 55.56 SPAD-502 meter readings, and 22.56%, respectively, was observed.

تأثير استخدام البكتيريا *Bacillus subtilis* والفطر *Ganoderma lucidum* والطحلب *Spirulina platansis* في تحفيز المقاومة الجهازية لصنفين من الباقلاء ضد فايروس موزائيك الفاصوليا الاصفر (BYMV)

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الخلاصة

هدفت الدراسة الى تقويم كفاءة البكتيريا *Bacillus subtilis* ومستحضر الفطر الريشي *Ganoderma lucidum* والطحلب *Spirulina platansis* على صنفين من نبات الباقلاء في اختزال التأثير السلبي لفيروس الموزائيك الاصفر على الباقلاء (BYMV) و تحفيز المقاومة الجهازية للنبات. اظهرت النتائج وجود تأثيرات معنوية للعوامل الحيوية المذكورة في خفض شدة الاصابة وزيادة في معايير النمو للنبات في حالات المعاملات المنفردة والتوليفات بينهم اذ حققت المعاملة بالعوامل الثلاثة مجتمعة مع الصنف برشلونة فرق معنوي في خفض شدة الاصابة بلغت 16.17% قياساً بالمعاملة المقارنة فضلاً عن الزيادة في معايير النمو المدروسة التي شملت ارتفاع النبات، مساحة سطح الورقة، نسبة الكلوروفيل، ونسبة العقد بقيم بلغت 42.33 سم و 44.77 سم^2 و 55.56 من قراءات جهاز تقدير الكلوروفيل "سباد" و 22.56 %، على التوالي.

الكلمات الافتتاحية: الطحالب، موزائيك، الفطر الريشي.

INTROUCTION

Faba bean (*Vicia faba* L.) is one of the most important crops in the legume family. Its seeds are used as a human food due to their high (33%) protein content (Graham and Vance, 2003). Legumes rank second after cereals in terms of economic importance, with 120 million hectares (12-15% of cultivated land) being devoted to growing them, worldwide. Their global production represents about 27% of the world's grain production (Al-Mayahi *et al*., 2014). Faba bean crop is susceptible to many viral diseases, including bean yellow mosaic virus (BYMV), one of the most 2007). This disease widely spread viruses on faba bean in Europe and the Arab world (Jones *et al*., is mechanically transmissible and is more dangerous as it is transmitted biologically by *Aphis fabae* and *Myzus persicae* Symptoms of infection may appear on all green parts of the shoot system, with pale yellowing of the veins occurring within seven days of infection, followed by yellow or green mosaic and newly emerging leaves with deformities and wrinkles (Athab , 2009).

The virus has a wide host range and infects growing wild plants such as chickpeas, wild radish, and beans (Al-Ani *et al*., 2011). Due to the importance of chickpeas as a food source for humans, several methods, including the chemical ones, have been adopted to protect the plant from infection, by combating insects that transmit the virus and by using biological agents to stimulate plant resistance mechanisms to resist attack by various pathogens, in general, and viruses in particular (Abbassy *et al.*,2014*;* Murphy *et al.*, 2003). Researchers have turned to using some fungi such as *Ganoderma lucidum* and *Spirulina* platensis alga to induce plant systemic resistance against plant pathogens (Ayed and Al-Fahad, 2018). For this, the present study aimed at determining the effect of these biological agents in reducing the severity of infection with bean yellow mosaic virus in broad bean.

MATERIAL AND METHODS

Faba bean plants showing symptoms characteristic to bean yellow mosaic virus (BYMV) infection were collected from Salah Al-Din province, Al-Shirqat district, Iraq and used as a source of the virus. The identity of the virus was determined based on symptomology, transmissibility by both mechanical and *Aphis faba* insect means.

Growth conditions:

Plants were grown in plastic trays with a diameter of 20 cm and a height of 25 cm. Trays were filled (1 kg per tray) with a mixture (1:1) of dry soil with vermiculite and sterelised with formalin (39%) at a rate of 4:1. The trays were then placed in the field.

Biological control agents:

Bacillus subtilis strain was obtained from the Plant Protection Department at Tikrit University's College of Agriculture. The bacterium was grown on nutrient agar medium in Petri plates and used in all relevant experiments in this study.

Mechanical transmission:

BYMV inocula were prepared by grinding fresh pieces of leaves taken from BYMV-infected plants showing typical symptoms of the virus were ground in pestle and mortar in the presence of phosphate-buffer (pH. 7). The resultant homogenate was then filtered through two pieces of cloth and the filtrate was used as an inoculum. Mechanical transmission was carried out by first dusting the upper leaf surface with carborundum as an abrasive and then the inoculum was gently rubbed against the upper surface with the thumb or a piece of cheese cloth. Excess inoculum was then washed off inoculated leaves with running tap water.

Biological agent application:

Nine treatments with biological control agents, either alone or in combination, were applied on to two BYMV-infected broad bean varieties, namely, Barcelona and a local cultivar. The nine treatments were as follows:

1- The bacterium *B. Subtilis* alone (B). A bacterial suspension (1×10^{-8} cell/cm^3).
2- The fungus *Ganoderma lucidum* alone (G.). Five grammes (5g) of the fungus spore powder were suspended in 1 litre of sterilised water.
3- The alga *Spirulina platensis* (S) A suspension of the dried ground of the Alga was prepared by suspending 5g of *S. platensis* powder into 1 litre of sterilised distilled water.
4- A combination of the bacterium ‘B’ and the fungus ‘G’ (B+G).
5- A combination of the bacterium ‘B’ and the alga ‘S’ (B+S).
6- A combination of the fungus ‘G’ and the alga ‘S’ (G+S).
7- A combination of the bacterium ‘B’, the fungus ‘G’, and the alga ‘S’ (B+G+S).
8- BYMV-inoculated control plants.
9- Untreated healthy control plants.

Each treatment was sprayed onto the test plants using a manual sprayer until the plants were completely wet. Plants received three treatments, the first was applied after the emergence of the fifth true leaf whereas the second was carried out one week later and the third was applied during the beginning of the flowering stage. Each treatment was repeated three times.

Treatment with *Bacillus Subtilis*:

Plants were sprayed with *Bacillus subtilis* after the emergence of the fifth true leaf.

Treatment with *Spirulina platensis*:

Plants were sprayed with *Spirulina platensis* after the emergence of the fifth true leaf.

Measuring growth parameters:

Plant height

Plant height (in cm) was measured as the distance from its contact point with the soil to the highest growing tip of the plant. This was done when the plant reached the stage of full maturity at the end of the experiment.

Leaf area

Leaf area (cm^2. $plant^{-1}$) was measured (Dovrnic, 1965) by taking three randomly selected leaves from each treatment during the vegetative growth stage at the true eighth leaf age and then weighed, and the weight of the disks from each leaf was recorded. The area was then calculated using the following equation as shown by.

Area of leaf (cm^2) = (known disk area x total disk weight)/ (known disk weight)

Calculating the percentage of set flowers:

The number of set flowers was calculated using the following formula:

The set flowers % = (Number of set flowers) / (Total number of flowers) × 100.

Fresh weight:

Assessing severity of BYMV infection:

The severity of infection was calculated by counting the number of infected plants based on the disease scale described in this study. The following equation (McKinney, 1923) was applied to calculate the severity of infection.

Severity of infection (%) = [(4 × number of heavily infected plants) + (2 × number of moderately infected plants) + (1 × number of lightly infected plants)] / (highest category) 5 × number of samples taken) × 100

Table 1. Disease severity scale for faba bean plants infected with bean yellow mosaic virus (BYMV) as compared with healthy control ones

Symptom (s)	Disease severity(Scale)
No symptoms (Healthy plants)	0
Slight leaf chlorosis	1
Leaf mottling	2
Leaf mottling and wrinkling	3
Leaf chlorosis, mottling, and wrinkling	4

Measuring chlorophyll content:

Chlorophyll content of leaves was measured using an SPAD-Chlorophyll meter. Three randomly chosen leaves were detached from the top, middle, and bottom parts of each plant. The relative chlorophyll content was estimated, and the arithmetic mean of the three readings was calculated.

Statistical analysis:

Experimental data resulting from the *randomised complete block design* were analysed using the SAS programme (1998) and the Duncan multiple range test was used to compare means (AL-Rawi and Khalafallah, 1980).

RESULTS AND DISSCUSION

Statistical analysis of results (Table 1) showed a variation in plant height amongst treatments. Significant differences were found between treatments, where the highest recorded height was that (45.16 cm) of the control healthy) plants , followed by that (41.33 cm) of the triple combination (B+G+S) treatment. However, the lowest height was that (27.33 cm) of the control infected plant treatment. As to the impact of variety, on plant height, there were no significant differences between the two varieties (Barcelona and the local variety). However, there were significant differences amongst treatments, where the highest plant height (42.13 cm) was observed with the Barcelona variety exposed to the triple combination treatment (B+G+S), followed by the Local variety with a mean height of 40.13cm.The superiority of the triple combination treatment is likely to be due to the additive effect of the three biological resistance agents in increasing plant systemic resistance to virus infection. Additionally, *Spirulina platensis* alga and *G. lucidum* fungus might `have served as a source of nutrients that have promoted plant growth.

Table 2. Effect of *Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina* alga on plant height (cm) of two varieties (Barcelona and a local one) of broad bean infected with bean yellow mosaic virus (BYMV)

Variety	Treatments									
	B	G	S	B+G	B+S	G+S	B+G+S	Control Infected	Control Helthy	Mean of varieties
Barcelona	39.00 Abc	39.00 abc	34.33 ef	38.66 Bcde	36.66 cdef	39.00 bcde	42.33 abc	27.66 g	43.33 A	37.77 a
Local variety	37.33 Bcd	37.33 bcd	40.33 bcd	35.00 Def	33.66 ef	31.00 fg	40.13 abc	27.00 g	44.00 A	36.37 a
Mean	40.66 Bc	40.66 bc	37.33 cd	36.83 D	35.16 d	35.00 d	41.33 b	27.33 e	45.16 A	

Different letters within column indicating of significant differences ($p<0.05$)

Similar increase in plant's resistance and height were reported before (Ayed. 2018). Additionally, it is highly likely that the bacterium *B. Subtilis* may through the excretion of many growth factors such as gibberellins, cytokinins, and auxins have increased plants' ability to absorb the nutrients present in the soil surrounding the root, which led to an increase in plant height and other growth traits (Soleiani *et al*., 2011; AL-Fahad *et al*., 2020). As to the effect of variety on virus resistance, its effect on all the traits studied may be attributed to the genetic constitution of the variety used that determines the hereditary potential of the variety tested (Mando *et al*., 2011).

Table 3. Effect of *Bacillus subtilis* (B), *Ganoderma lucidum* (G), and *Spirulina* alga (S) on infection severity (%) of two varieties of broad bean infected with bean yellow mosaic virus

	Treatments									
Variety	B	G	S	B+G	B+S	G+S	B+G+S	Control Infected	Control Helthy	Mean of varieties
Barcelona	34.43 Bc	32.74 bcd	36.88 b	27.23 cd	30.81 bcd	32.01 bcd	16.17 e	95.54 a	0.00 f	33.45 a
Local variety	29.10 Cd	37.53 b	33.77 bcd	29.18 cd	31.33 bcd	26.56 d	17.06 e	96.533 a	0.00 f	33.98 a
Mean	31.76 Bc	35.13 b	35.32 b	28.21 c	31.07 bc	29.28 c	16.62 d	96.03 a	0.00 e	

Different letters within column indicating of significant differences (p<0.05)

It is clear from the results obtained herewith (table 3) regarding the effect of biocontrol agents (*Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina* alga) tested either individually or in combination on the severity (%) of infection of two varieties (Barcelona and a local one) of broad bean that significant differences in the mean of infection severity was evident. As expected, the treatment "Control–healthy" for the two varieties (Barcelona and the local one) gave the lowest (0.00%) mean of severity of infection . In contrast, the severity of infection was the highest (95.54%) in the Barcelona variety and the local one (96.533%), with a mean of 96.03% and no significant difference between the two. Notably, the minimum severity of infection (16.17 and 17.06%) was reported for the triple biocontrol agent treatment in both Barcelona and the local varieties (respectively), with a mean of 16.62% but with no significant difference between the two. In contrast, the other treatments with the biocontrol agents taken either individually or in combinations of two was associated with a degree of severity of infection in the two varieties lying in between the two extremes with no significant difference amongst most of them.
However, there was no significant difference between the double combination and the control treatments. The when used as a healthy control treatment.

The reduction in the severity of infection observed on plants treated with these biocontrol agents particularly when triple combinations were used may be attributed to the antagonistic activity of the agents used. The bacterium *B. Subtilis* is known to excrete antibiotics that can suppress the pathogen growth (Han *et al.*, 2005). Additionally, the bacterium is capable of producing degradative enzymes, such as chitinase, B-1,3 glucanase, and peroxidase (Harsh *et al.*, 2009; EL-Borollosy and Oraby, 2012) and all these may contribute to reducing the severity of infection. As to the alga *S. Platansis*, it is likely that it produces many antiviral compounds that may interfere directly with the infection process or indirectly by inducing plants to resist the pathogen (Usharani *et al.*, 2015). Similarly, the fungus *G. lucidum* may reduce the severity of infection due to compounds that have an antiviral effect, primarily proteins, sterols, and triterpenoids (Mehta and Jandaik, 2012: Ayed and Al-Fahad, 2018).

Table 4. Effect of *Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina* alga on chlorophyll percentage (%) of two varieties (Barcelona and a local one) of broad bean infected with bean yellow mosaic virus

Variety	Treatments									
	B	G	S	B+G	B+S	G+S	B+G+S	Control Infected	Control Helthy	Mean of varieties
Barcelona	41.13 cde	46.66 abc	52.76 bcd	48.50 bc	49.20 abc	49.33 Abc	55.56 abc	29.00 e	61.06 a	47.87 a
Local variety	47.36 abc	42.13 bc	49.26 cd	52.26 abc	47.76 bc	48.36 Bc	55.16 abc	31.93 de	59.26 ab	47.99 a
Mean	44.25 c	49.40 bc	44.01 c	50.38 bc	48.48 bc	48.85 Bc	55.36 ab	30.46 d	60.16 a	

Different letters within column indicating of significant differences (p<0.05)

The results displayed in Table 4, indicated that infection with BYMV significantly reduced chlorophyll content (29.00% and 31.93 with a mean of 30.46) in the two varieties (Barcelona and the local one, respectively) as compared to that (61.06% and 59.26% with a mean of 60.16%) of the corresponding healthy control plants. As to the effect of treating BYMV- infected broad bean plants with the three biocontrol agents, namely, *Bacillus subtilis* (B), *Ganoderma lucidum* (G), and *Spirulina* alga (S) either individually or in combinations of two or three indicated that chlorophyll content was significantly reduced as compared to the corresponding means mentioned above for the healthy control plants in the two varieties. The triple combination treatment gave the highest (55.56 and 55.16 with a mean of 55.36) chlorophyll content for the two varieties (Barcelona and the local variety, respectively) when measured at 55.56 Spad, yet no significant difference was found between estimates in the two varieties. The chlorophyll content estimates for plants treated with the biocontrol agents (either individually or in combinations of twos) in both varieties lied in between the two extremes with no significant differences were found amongst them.

The beneficial effect of the use of *Bacillus subtilis* (B) in terms of increasing chlorophyll content is likely to be due to its ability to provide the host plant with some nutrients and to stimulate systemic resistance that limit virus reproduction and consequently limit chlorophyll degradation (Al-Samra'i Sahar, 2018). Similarly, the alga *S. platansis* role in increasing chlorophyll content may be attributed to the trimethylated betaine, a modified amino acid consisting of glycine with three methyl groups serving as a methyl donor for various metabolic pathways and the magnesium it provides the host plant , considered sources of nitrogen that increase chlorophyll levels (Kuwada, 2006; Salmean *et al.*, 2015). Butler and Hunter (2006) confirmed through their results that the importance of adding marine algae extracts is due to the elements they contain that increase plant growth and improve plant health and productivity. The role of reishi mushrooms has been shown (Gao *et al*., 2003; Mehta and Jandaik, 2012; Ayed. 2018) to be due to increasing the effectiveness of plant vitality, resistance, and chlorophyll content.

Table 5. Effect of *Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina* alga on leaf area (cm^2) of two varieties (Barcelona and a local one) of broad bean infected with bean yellow mosaic virus

	Treatments									
Variety	B	G	S	B+G	B+S	G+S	B+G+S	Control Infected	Control Helthy	Mean of varieties
Barcelona	19.80 def	15.25 gh	21.56 de	23.92 cd	27.95 c	15.79 Fg	40.77 b	43.18 a	11.16 Hi	24.60 a
Local variety	19.22 efg	14.87 fg	18.63 fge	21.83 de	19.01 efg	14.64 Gh	37.66 b	40.01 b	9.55 I	21.82 b
Mean	19.51 E	20.06 de	15.09 f	22.87 cd	23.48 c	15.21 F	39.21 b	43.09 a	10.35 G	

Different letters within column indicating of significant differences (p<0.05)

Statistical analysis of the results shown in Table 5 showed significant variation in leaf area amongst different treatments with the biocontrol agents (*Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina* alga) applied either individually or in combinations in twos or threes. Obviously, The highest mean (43.18 cm^2) of leaf area was that of the healthy control Barcelona followed by that (40.01 cm^2) of the healthy control local variety with a mean of 43.09 for the two varieties, while the lowest mean was that (9.55 cm^2) of the infected local variety and that (11. 16) of the infected Barcelona variety with a mean of 10.35 cm^2 for the two varieties. Control treatment. As for the varieties, there was a significant difference between them in the mean leaf area, where the first variety (Barcelona) had the highest mean (24.60 cm^2) followed by the local variety with a mean area of 21.82 cm^2. The other treatments with the biocontrol, agents taken either individually or in combinations of twos or threes led to leaf areas lying in between the two extremes in both varieties with the triple treatment (B+G+S) in both varieties being the highest (40.77 and 37.66 cm^2) in leaf area for the two varieties (Barcelona and the local variety, respectively) with a mean of 39.21 cm^2.

The significant reduction observed in the growth of the control BYMV-infected broad bean plants is likely to be due to increased plant respiration and carbohydrate consumption associated with infection (Mehrotra, 2017). Additionally, root damage may hinder nutrient transfer, resulting in plant stunting, decrease in dry mass, and, consequently, decrease in leaf area (Al-Eidani, 2010).

The increase in leaf area resulting from the use of biocontrol factors and nutrients is likely to be due to the possible stimulation of plant growth by increasing the availability of nutrients and enhancing its resistance to pathogens through the production of salicylic acid (VanLoon and Bakker, 2003 ;Bargabus *et al.*, 2004) The alga *S. platansis* and the fungus *G. lucidum*, seem to have caused the increase in leaf area as a result of being a source of nutrients and growth regulators such as auxins and gibberellins known to increase nutrient absorption and cell division rates in addition to their ability to activate enzymes that stimulate plant growth and disease resistance (Al-Fahd, M. A. and Al-Jumaili S. A, 2011; Jensen, 2004) which collectively culminate in an increase in vegetative growth and leaf area.

Table 6. Effect of *Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina* alga on percentage (%) of fruit setting in two varieties (Barcelona and a local one) of broad bean infected with bean yellow mosaic virus

	Treatments									
Variety	B	G	S	B+G	B+S	G+S	B+G+S	Control Infected	Control Helthy	Mean of varieties
Barcelona	16.76 de	11.23 h	18.00 d	16.23 De	15.85 de	13.53 Fg	22.56 c	28.46 A	8.43 i	16.78 a
Local variety	12.37 gh	10.50 h	11.43 gh	13.61 fg	14.82 ef	12.68 Fgh	20.66 c	25.95 B	7.83 i	14.43 b
Mean	14.56 c	10.86 e	14.71 c	14.92 c	15.33 de	13.10 D	21.61 b	27.21 A	8.13 f	

Different letters within column indicating of significant differences (p<0.05)

The results displayed in Table 6 show that the healthy control plants for the two varieties (Barcelona and the local one) had the highest percentage (28.46 and 25.95%, respectively) with a mean of 27.21% of fruit setting. In contrast, the BYMV-infected broad bean control plants of the two varieties (Barcelona and the local one) had the lowest percentage (8.43 and 7.83%, respectively)و with a mean of 8.13%, of fruit setting. The cultivar treatment gave the highest rate of varieties at (16.78%). Noticeably, all other biocontrol treatments (tested either individually or in combinations of twos or threes), led to an intermediate percentage values of fruit setting with the triple combination (*Bacillus subtilis*, *Ganoderma lucidum*, and *Spirulina* alga) treatment having the highest percentage (22.56% and 20.66%) of fruit setting in both varieties amongst all. In contrast, the fungus (*G. lucidum*) treatment in both varieties gave the lowest fruit setting percentage (11.23 and 10.50%, respectively) for both varieties with a mean of 10.86%.

The noticeable increase in fruit setting is likely to be due mainly to the beneficial constituents derived from the biological factors. The bacterium B. *subtilis* may have caused an increase in the percentage of flowers and fruit setting through the plant hormones, auxins (e.g. indole acetic acid, IAA) and Gibberellins (GAs) it may excrete (Al-Eidani, 2010). As to the observed increase in fruit setting resulting from the addition of *S. platansis* alga and G. *lucidum* fungus, it is feasible to assume that to their containing many metabolic compounds such as polysaccharides and ganodermanin produced by *G. lucidum* fungus and *S. platansis* alga (Sivanandhan *et al.*, 2017 ;

Gao *et al.*, 2003) which are antioxidants for many plant-pathogenic fungi and viruses, which led to stimulating plant growth and thus increasing the percentage of flowers setting.

CONCLUSION

This study concludes that Barcelona verity is more resistance than local verity, the biological factors also showed that they are active in reducing disease severity wherever they are used in particular or combined.

CONFLICT OF INTEREST

There is no conflict of interest during investigating this study.

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