

## EFFECT OF BROAD BEAN MOTTLE VIRUS INFECTION ON FABA BEAN NODULATION AND EFFICIENCY OF N<sub>2</sub>-FIXATION

[3]

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### ABSTRACT

Plant growth and root nodules of infected Faba bean plants with broad bean mottle virus were significantly reduced in plant height, leaf area, dry matter, number and size of nodules in comparison with healthy ones. As well Leghaemoglobin content and Nitrogenase activity were reduced in infected nodules at 45 days after BBMV inoculation. The amount of N<sub>2</sub>-fixed as expressed by acetylene reduction was reduced considerably by 38.8%. Nitrogen content as an indicator for the efficiency of N<sub>2</sub>-fixation was reduced in shoots, roots and root nodules of infected nodulated plants as compared with healthy nodulated ones. Differences between bacteroids in healthy and in BBMV infected cells were no longer apparent. These differences include: (i) a decrease in endocytotic and exocytotic vesiculation on the membrane envelope of the bacteroids and on plasma membrane of bacteroidal cells, (ii) a decrease in number of vesicles in the space between the bacteriod and the membrane envelope. The possible significance of these changes relative to the decreased efficiency of N<sub>2</sub>-fixation was discussed.

**Keywords:** Faba bean plants, Rhizobia, Broad bean mottle virus, Leghaemoglobin, Nitrogenase, N<sub>2</sub>-fixation.

### INTRODUCTION

Rhizobial root nodules of legumes plants appear to be affected by viruses, for it is known that virus diseased legumes plants produce smaller, fewer and less effective root nodules than healthy

plants (Tu, 1977; Guy *et al* 1980 and Ishac *et al* 1983). It also is known that the virus is actually present in root nodule cells of infected plants (Tu, 1977). However, little is known about the effect of virus infection on the Rhizobia *in situ*.

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(Received September 7, 2005)  
(Accepted November 14, 2005)

Yield and plant weight reduction in plants infected by soybean mosaic virus (SMV) (Tu, 1973; Ishac *et al* 1983) and bean pod mottle virus (BPMV) (Tu, *et al* 1970) have been attributed both to photosynthetic reduction and reduced nodulation. Although reduction of nodule size, number and fresh weight have been showed in SMV-infected soybean plants (Ishac *et al* 1983). It is not known if the efficiency of N<sub>2</sub>-fixation by those nodules has been impaired-variations in size and numbers of nodules which may not be important factors in plant growth without knowing their efficiency in N-fixation. Increased efficiency could offset any deleterious effects of reduction of size and number of nodules.

Broad bean mottle virus (BBMV) is belonging to genus bromoviruses. It transmits by mechanical inoculation, grafting and insect; but not transmits by seeds. The virion is isometric, non enveloped, 26 nm in diameter and rounded (Brunt *et al* 1996).

Few reports have shown the effect of Broad bean mottle virus (BBMV) on growth retardation, leghaemoglobin content and Nitrogenase activity. Consequently, the effect of BBMV infection on the efficiency of N<sub>2</sub>-fixation and ultra-structure of bacteroidal cells in root nodules were not yet study in detail. Therefore the present study aimed to illustrate such effect.

## MATERIAL AND METHODS

An isolate of BBMV and strain of *Rhizobium leguminosarum* were obtained from Agric. Microbiology Dept., Fac. of Agric., Ain shams Univ. The Faba bean cv. Giza 143 was used as suscepti-

ble host to BBMV infection and *R. leguminosarum* inoculation.

The experiment was done in pots under green house conditions using sterilized soil in complete randomized design. Seeds of Faba bean were treated with cell suspension (10<sup>10</sup> cell /ml) of *R. leguminosarum* using 2 ml /one gram of seeds. BBMV was mechanically inoculated at first trifoliolate leaf stage. The treatments of experiment as follows:

- I. Uninoculated, healthy plants.
- II. Inoculated plants with *R. leguminosarum*.
- III. Mechanically inoculated plants with BBMV.
- IV. Inoculated plants with *R. leguminosarum* and infected with BBMV.

Number of the plants for each treatment was 50 plants (5 in each pot). The parameters and representative samples from leaves, shoots, roots, and root nodules were taken for each treatment, and the following parameters were estimated:.

- 1- Virus concentration in leaves, roots and nodules was biologically assayed using *Chenopodium amaranticolor* as local lesion host, at 15, 30, 45, 55 days post BBMV inoculation (Ross, 1967). An equal weighty top leaves, roots and nodules of BBMV inoculated plants were extracted in 0.01 M phosphate buffer, PH 7.0(1ml buffer per gram tissues). The 10<sup>-1</sup> dilution as well as the crude extract were mechanically inoculated on half leaves of *Ch. Amaranticolor* using Latin square design. Local lesions were counted 7 days after virus inoculation.
- 2- The morphological (plant height and leaf area) and chemical parameters were done on ten plants from each treatment. Leaf area was calculated

by the relationship between weight of calico paper of about 10x10 cm and the weight of the unit area. Number, size and dry weight of nodules were calculated. Size of nodules were measured by displacement of water.

- 3- Assessment of nitrogenase activity in nodules was determined according to **Dart and Harris (1972)**. Two ml of gas withdrawn from syringes contained 0.2 gm fresh nodules and injected with acetylene and incubated at 30°C for one hour and assayed for ethylene concentration using UNICAM gas liquid chromatography model 104 fitted with dual flame ionization detectors and a 150cm long and 0.4 cm stainless steel column fitted with 10% of Na<sub>3</sub> PO<sub>4</sub> on spherosil XOP 075, 100–200 mesh prepared by Peknen Elmar Company, Bodensee Werks F.P. Germany. Standard curve of the used ethylene was made by injection 1200 µl C<sub>2</sub>H<sub>4</sub>. A linear relation between ethylene concentrations and ethylene peak heights was obtained for each attenuation. The results were presented as µ moles C<sub>2</sub>H<sub>4</sub> / weight / h. multiplying by the conversion factor 3 gives the equivalent µ moles of N<sub>2</sub>-fixed. The micromoles of N<sub>2</sub>-fixed multiplied by 28 gives micrograms N<sub>2</sub>-fixed. It was calculated as micrograms /pot / h.
- 4- Leghemoglobin content in nodules was determined according to **Tu et al (1970)**. One gram of frozen nodules was homogenized in 5 ml of 0.1N KOH and centrifuged at 7500 rpm for 10 min. 1.5ml of supernatant was mixed with 1.0 ml distilled water and 0.5 ml of 2N KOH. The 0.1gm of Na<sub>2</sub>S<sub>2</sub>O<sub>2</sub>. After 10 min from mixing,

optical density of leghemoglobin was measured at optical density 537, 557 and 577 nm. The O.D. for leghemoglobin was calculated using the formula of  $OD_{557} = \frac{1}{2} (OD_{537} + OD_{577})$ .

- 5- Ultrastructure of bacteroidal cells was examined according to method described by **Abdel-Ghaffar (1994)**. Nodule discs about 1 mm were cut and fixed for 4 h. in 0.08 M cacodylate buffer, pH 7.4 containing 5% glutaraldehyde and 4% paraformaldehyde. The fixed specimens were washed three times at half-hour intervals with 0.1 M cacodylate buffer, pH 7.4 containing 3% sucrose. The samples were post fixed in 1% osmium tetroxide dissolved in a solution of 0.1 M cacodylate buffer and 2% sucrose. Following three half-hour washes in 0.1 M cacodylate buffer-3% sucrose, pH (7.4).The samples were dehydrated in alcohol series, sequentially followed by propylene oxide plus epoxy resin (1:1, v/v) and finally embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate then lead citrate, and examined in Zeiss 10 C transmission electron microscope at National Research Center, Giza, Egypt.

## RESULTS

BBMV concentration was significantly reduced in leave, nodule and roots of infected plants (IV) in comparison with non infected nodulated plant (Treatment III) (Table 1), when estimated as a local lesions number of *Ch. amaranticolor*. Generally BBMV concentration was increased after 45 days post inoculation in leave, root and nodules.

Table 1. Effect of *R. leguminosarum* inoculation on BBMV concentration as indexing by local lesion on *Ch. Amaranticolor*

Treatment periods	Leaves				Roots				Nodules	
	III		IV		III		IV		IV	
	10 <sup>-1</sup>	mean	10 <sup>-1</sup>	mean	10 <sup>-1</sup>	Mean	10 <sup>-1</sup>	mean	10 <sup>-1</sup>	Mean
25	14.35	12.43	15.15	12.20	12.50	10.63	11.25	8.25	4.75	3.58
35	20.75	17.75	17.10	14.25	13.75	11.50	12.65	9.95	7.95	6.10
45	19	18.75	20.50	15.87	11.25	14	8.12	12.6	13.25	12
55	17.25	14.88	12.25	11.50	15.75	14.03	14.2	17.85	10.70	8.58
Mean	17.83	15.95	16.25	13.57	13.31	11.54	11.56	9.23	9.16	7.56

III - infected with BBMV only

IV - inoculated with *R. leguminosarum* and infected with BBMV

LSD	Treatments	period	Treatments	period
5%	1.32	1.75	1.02	0.95
1%	2.35	3.20	1.95	1.75

Data of plant height, and leaf area were recorded in (Table, 2). It was found that, infected Faba bean plants with BBMV gave significant reduction in plant height. The plants inoculated with *R. leguminosarum* (II) showed the highest increase in plant height in comparison with control after 55 days in comparison with plants infected with BBMV(III) and those inoculated with *R. leguminosarum* and infected with BBMV(IV). The rate of such increase was found to be (Table, 2) 21.6; 41.7; 26.5 and 21.6% respectively.

Concerning leaf area, it was noticed that plants infected with BBMV (III) showed significant reduction of leaf area in comparison with control (I), inoculated *R. leguminosarum* (II) and infected with BBMV (IV) at different periods of plant growth. Such reduction of leaf area of treatment III compared with treatment IV was found to be 50, 65, 21 and 25% respectively.

Results in Table (3) showed that plants infected with BBMV(III) and it which inoculated with *R. leguminosarum* (IV) had decreased in dry matter. The reduction was found to be significant in first and fourth periods compared with control plants (I) and (II) respectively. The total nitrogen percentage of broad bean shoots was found increased non-significantly in the case (IV) of infected plants with BBMV (III) and it which inoculated with *R. leguminosarum* in comparison with control plants (I) and a bacterized plants (II) respectively. It was found that, plants bacterized and inoculated with BBMV increased non-significantly at the all periods (Table 3). BBMV- infection affected on nodulation and nitrogen fixation. It was found significant reduction in number, size and dry matter of nodules in infected plants with BBMV (IV) compared with non-infected one (II). Such increase was found in the rate of 43; 45 and 93% (Table 4).

Table 2. Effect of **BBMV** on plant height and leaf area of Faba bean plants

Days post -BBMV infection	Plant height (cm)				Leaf area mm <sup>2</sup>			
	I	II	III	IV	I	II	III	IV
25 days	28.75	32.50	26.50	29.75	65.5	180.5	63.21	95.10
35 days	32.50	38.50	30.75	33.50	110.80	228.5	95.15	157.20
45 days	35.70	41	30.50	33.95	152.7	243.5	145.30	175.30
55 days	37	45	31.75	35.75	185.4	259.3	145.4	182.20
Mean	33.49	39.25	29.87	33.24	128.1	217.95	112.26	152.70

I – healthy plants (control)

II – healthy plants and inoculated with *R. leguminosarum*.

III – infected plants with BBMV.

IV – inoculated plants and infected with BBMV.

Mean from 10 plants

LSD	Treatment	period	Treatment	period
5%	1.25	1.75	3.75	4.25
1%	2.10	2.65	5.25	6.62

Table 3. Effect of **BBMV** infection on dry matter and total nitrogen content of Faba bean plant

Days post -BBMV infection	Dry matter * (g plant shoot)				Total nitrogen ** (mg/100g dry matter)			
	I	II	III	IV	I	II	III	IV
25 days	1.30	1.35	1.04	1.12	1.95	2.25	2.05	2.08
35 days	1.37	1.50	0.95	1.21	2	2.75	2.53	2.56
45 days	1.59	1.79	1.29	1.49	3.25	4.05	3.41	3.82
55 days	1.71	1.86	1.53	1.51	3.56	4.65	4.02	4.30
Mean	1.49	1.63	1.20	1.33	2.69	3.43	3	2.19

\* Dry matter content calculated as mg/plant shoot

\*\* Total nitrogen content percentage calculated as mg/100g dry matter.

LSD	Treatments	/	Treatment
5%	0.25		0.65
1%	0.40		0.80

It was clear from data represented in Table (4) that infected nodules had considerable increase in total nitrogen percentage in comparison with healthy nodules (II) such increase was found to be 4, 10, 80 and 5% after 25, 35, 45 and 55 days.

Nodules of BBMV- infected plants showed significant reduction in both leghaemoglobin content and nitrogenase activity in comparison with those formed from healthy plants (Table 4). The percentage of reduction in leghaemoglobin content was found to be 17, 15, 52 and 33% at the 25, 35, 45 and 55 days respectively. The reduction in nitrogenase activity was found to be 28, 40, 46 and 41% respectively after the same previous periods.

Data represented in Table 4 showed that, both treatment (II) and (IV) gave the highest amount of  $N_2$ - fixed at 55 days. At the same period significant reduction in the quantity of the  $N_2$ - fixed was observed infected nodulated plants(IV) by the rate 25, 42, 46 and 39 % comparison with healthy nodulated plants (II).

The micrographs that are presented, show differences between healthy and BBMV-infected bacteroidal cells, which are most noticeable at early stages. Figure(1) is a low magnification micrograph of a BBMV-infected bacteroidal cells. The presence of BBMV particles in the BBMV-infected bacteroidal cell in readily discernible Figure (2). One obvious and important difference in the greater space between the bacteria and the ME in healthy cells than in BBMV-infected cells (compare Figure 3 with Figure 4): this may be indicate Leghaemoglobin is located in the space between the symbiotic bacteria and the ME in the bacteroidal cells. In these spaces a network of lightly

stained fibrillar material is clearly observed in the bacteroid cells in healthy root nodules.

A second difference is the vesicles in the space between the bacteria and the ME in healthy bacteroidal cells are more numerous than those in cells of plants infected with BBMV (Figure 3 and 4). Assuming that the vesicles are related to endocytosis and exocytosis, then a higher vesicle density in bacteroidal cell healthy would indicate a higher movement of material between the bacteria and the host cytoplasm. A third difference is healthy cells have a much larger amount of unevenly thick amorphous layer of electron-opaque material lining the inside of the ME (Figure 4 asterisk). It is these electron-dense portions of the ME the protrusions are formed (Figure 4). A fourth difference is in the concentration of vesicles associated with the plasma membrane of the bacteroidal cell. These results suggest a higher rate of material movement in and out of the bacteroidal cells.

## DISCUSSION

In the present study, biological assay indicates that inoculation of broad bean plants with *R. leguminosarum* clearly reduced concentration of BBMV in leaves and roots. This reduction may be due to the increase in plant growth, chlorophyll content of nodulated infected plants (El-Dougdoug, 1982) and have decrease in susceptibility especially during the latter stage of infection(55 days). Increased pigment in less susceptible IR<sub>20</sub> of rice cultivar during the later stages of infection was reported by (Stidanar, *et al* 1978) who added that the cytokinin activity in IR<sub>20</sub> was 69 % higher than in healthy plants.

Table 4. Effect of BBMV infection on nodulation, leghaemoglobin, nitrogenase activity and N<sub>2</sub>-fixed of Faba bean plants

	No. of nodules per plant *		Size of nodules per plant *(cm)		Dry matter of nodules *		Total nitrogen **		Leghaemoglobin content ***		Nitrogenase activity ****		N <sub>2</sub> -fixed *****	
	II	IV	II	IV	II	IV	II	IV	II	IV	II	IV	II	IV
25	14.75	10.00	0.125	0.035	0.1750	0.0035	3.025	3.157	0.75	0.62	35.52	25.60	352.70	265.25
35	24.50	14.75	0.35	0.15	0.4525	0.125	3.925	4.325	0.94	0.80	44.50	26.75	420.25	245.75
45	35.0	13.55	0.450	0.170	0.4256	0.0562	2.750	4.950	1.15	0.55	45.70	24.50	425.20	230.25
55	23.15	17.25	1.025	0.725	0.6225	0.0352	2.265	3.465	0.42	0.28	38.25	22.75	345.26	210.75
Average	24.35	13.88	0.488	0.270	0.4189	0.0269	2.991	3.974	0.815	0.563	41.010	24.90	385.85	238.0

\* Average from 5 plants

\*\* Total nitrogen as gm/100g dry matter nodules

\*\*\* Leghaemoglobin content calculated as optical density /g fresh weight

\*\*\*\* Nitrogenase activity measured by acetylene reduction

\*\*\*\*\* N<sub>2</sub>-fixed was calculated as  $\mu$  gm/pot/hr.

LSD

5% 2.85 0.125 0.175 0.251 0.125 5.25 25.25

1% 4.25 0.193 0.243 0.401 0.275 8.37 40.27

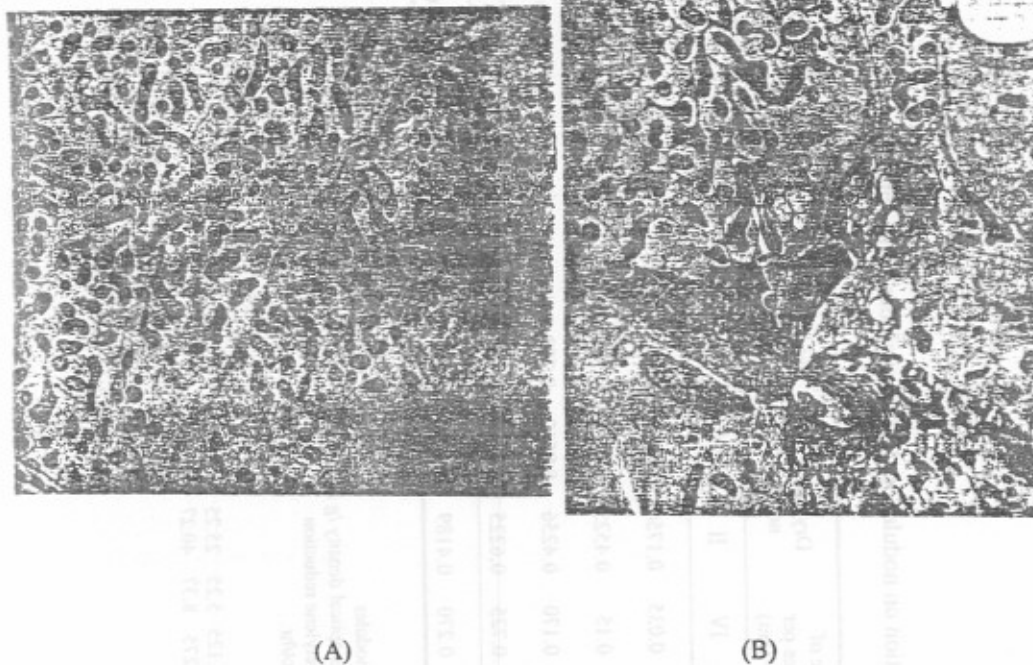


Figure 1a. A portion of a bacteroidal cells of rhizobial inoculation in healthy root nodule, with clear view of both vesicles and protrusions on ME of the bacteroid. Note there is a large space between the EM and the bacterium. Figure 1b: portion of root nodule cells infected with BBMV and rhizobial infection threads. Showing the presence of extensive of EM, elongation of mitochondria and virus aggregate.



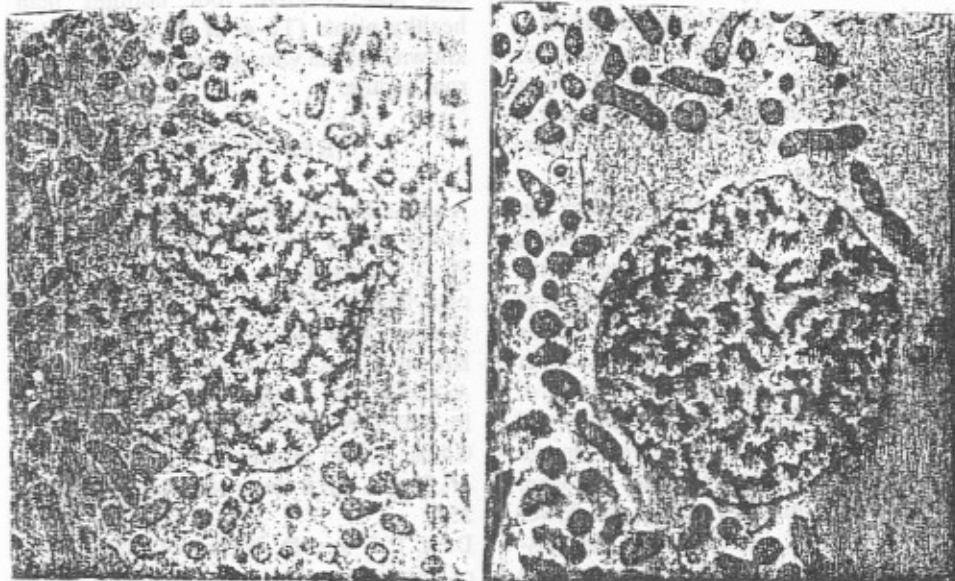


Figure 2a. Higher magnification of figure 1b showing details of bacteroids. The space between the bacteroid and the ME is very narrow. Figure 2b. A portion of a bacteroidal cell at the early stage of rhizobial infection in healthy root nodule showing the presence of vesicles and protrusions on the ME of the bacteroid. There is a large space between the ME and the bacterium.

The highest BBMV concentration in Faba bean root nodules was found 45 days post-infection, this gives evidence that BBMV could multiply inside the nodules. Tu (1975) reported the same conclusion using thin sectioning and freeze fracturing techniques in scanning electron microscopy. On the other hand the decrease in BBMV concentration in roots may be explained by the reduction in water uptake. A correlation between reduction in water uptake and increase of root resistance was found by Lindsey and Gudauskas, (1974).

Plant height and leaf area were significantly reduced in BBMV infected plants and inoculated with *R. leguminosarum*. This reduction may be the cause of subsequent reduction in photosynthetic activity (Orellana *et al* 1980). The latter is strongly correlated to the content of chlorophyll. Infected nodules were found to contain significantly higher percentage of both dry weight and total nitrogen. This increase may be due to the lower assimilation of carbohydrate in N<sub>2</sub>-fixation process in diseased nodules since the number of bacteroidal cells was reduced in comparison with healthy ones (Tu, 1977).

Roots of infected Faba bean plants were contained lower dry weight and higher percentage of total nitrogen than both nodulated and non-nodulated healthy plants. Cowpea plants infected with southern bean mosaic virus were found to increase total nitrogen (Mall *et al* 1980; Singh *et al* 1980 and El-DougDoug, 1982). A considerable decrease in dry matter content of southern bean mosaic virus was found by (Singh and Singh, 1980). It could be concluded that BBMV is a significant factor through its effect on plant growth

and the Faba bean-Rhizobium symbiosis (Orellana *et al* 1980 and El-DougDoug, 1982)

Rhizobial root nodules of broad bean plants appear to be affected by BBMV, for it is known that BBMV. Diseased broad bean plants produce smaller, fewer and less effective root nodules than healthy plants (Tu *et al* 1970). It is also known that the virus is actually present in root nodule.

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مجلة اتحاد الجامعات العربية للدراسات والبحوث الزراعية ، جامعة عين شمس ، القاهرة ، ١٤(١) ، ٤٧-٥٨ ، ٢٠٠٦

## تأثير الإصابة بفيروس الفول البلدي على تكوين العقد البكتيرية وكفاءة تثبيت النيتروجين في نباتات الفول البلدي

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بمقارنتها بالنباتات السليمة، بالإضافة للتباين الملحوظ في التركيب التشريحي العقد البكتيرية في النباتات المصابة عند فحصها بالميكروسكوب الإلكتروني بالمقارنة بعقد النباتات السليمة. وكذلك وجد أن محتوى ليجهيموجلوبيين والنشاط النيتروجيني في العقد البكتيرية ينخفض بعد ٤٥ يوم من الحقن بالفيروس، كما قلت كمية النيتروجين المثبت بنسبة ٣٨%. كما لوحظ نقص تثبيت النيتروجين في المجموع الخضري، الجذور، العقد الجذرية (البكتيرية) في النباتات المصابة بالفيروس.

تتأثر العقد البكتيرية في النباتات البقولية بالإصابة الفيروسية. فقد أظهرت الدراسات نقص ملحوظ في عدد وحجم العقد البكتيرية في النباتات البقولية المصابة بالفيروسات، يتناول هذا البحث دراسة تأثير فيروس تبقع الفول البلدي على تكوين العقد البكتيرية وكفاءة تثبيت النيتروجين في نباتات الفول. وتتلخص نتائج الدراسة فيما يلي: يتأثر نمو نباتات الفول والعقد الجذرية في نباتات الفول المصابة بفيروس تبقع نباتات الفول حيث يختزل طول النبات، مساحة الورقة، الوزن الجاف، عدد وحجم العقد البكتيرية

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