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Research Article

The use of rhizobium and mycorrhizae in soil containing rhizobiophage to improve growth and nodulation of cowpea

Yasmine Allam[1]*, Gehan Amin[1], Gamal Abdel Fattah[2], Abeer Hashem[3], Dilfuza Egamberdieva[4], Elsayed Fathi Abd_Allah[5], Gamal El-Didamony[1]

[1]Zagazig University/Faculty of Science – Dept. of Botany and Microbiology, P.O. Box 44519 – Zagazig – Egypt.
[2]Mansoura University/Faculty of Science – Dept. of Botany, P.O. Box 35516 – 11432 – Mansoura – Egypt.
[3]King Saud University/College of Science – Dept. of Botany and Microbiology, P.O. Box 2460 – Riyadh – 11451 – Saudi Arabia.
[4] National University of Uzbekistan/Faculty of Biology, 100174 – Tashkent, Uzbekistan.
[5]King Saud University/College of Food and Agricultural Sciences – Plant Production Dept., P.O. Box 2460 – 11451 – Riyadh – Saudi Arabia.
*Corresponding author <yasmineallam332003@gmail.com>

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ABSTRACT: The interaction between leguminous plants and rhizosphere microorganisms is essential because it can either enhance or inhibit the beneficial effects of individual species. Phages are one of the biotic factors with a negative impact on the beneficial bacteria in soil rhizosphere. In the current study, phage showed lytic activity against *Bradyrhizobium* sp. *Vigna (tal16)* with an icosahedral head at a 43.44 nm diameter and a long non–contractile tail, measuring 99.85 nm. This phage belongs to the *Siphoviridae* family, found in the Met El–Ez area of Dakalia Governorate in Egypt. The results revealed that the presence of phage in soil affected nodulation and growth parameters. Mycorrhizal inoculation aggravated the negative effects of this phage. Cowpea grown in soil containing phage VB_BrV_SD4 showed a reduction in the nodule number, nitrogenase activity, and total N of 40–50 %; however, mycorrhizal inoculation augmented this negative effect with a reduction percentage to 20–28 %. Mycorrhizal inoculation also improved total chlorophyll, carotenoids, legume amount, and the seed protein content.

Keywords: symbiosis, interaction, bacteriophage, agricultural microorganisms, nitrogen fixation

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important legume crop whose yield depends on the legume-specific strain of rhizobium that fixes N into ammonia, a crucial component in chlorophyll and protein synthesis (Singh et al., 2007). The high growth rate of legume plants depends on N fixation by rhizobium. Nitrogen builds amino acids, purines, pyrimidines, producing proteins and nucleic acids and building enzymes (Muleta, 2017).

A vascular arbuscular mycorrhiza (VAM) ("fungus – root") is an endophytic, biotrophic, mutualistic symbiosis in which arbuscular mycorrhiza (AM) fungi are coenocytic, with hundreds of nuclei in their hyphae and spores (Zhu and Yao, 2004). Arbuscular mycorrhizal fungi (AMF) can absorb immobile nutrients in plants, particularly phosphorus (Abdel-Fattah et al., 2014; van der Heijden et al., 2015). AMF reduces the use of chemical fertilizer by up to a half (Rai, 2006).

The symbiotic process between legumes and bradyrhizobium and VAM promotes plant growth and nutrient absorption, while inhibiting pathogen activity (Artursson et al., 2006). Mycorrhiza absorbs phosphorus (P) and uses it in energy metabolism, triggering N fixation by rhizobium (Whiting and Dilworth, 1974), nodule formation, and plant growth (Muleta, 2017). This dual colonization increases the growth and mycorrhizal inoculation rate as well as nodulation parameters, N_2 fixation, and N_2 and the P content in pea compared with nodulated legume plants only (Stancheva et al., 2006; Mugabo and Bhople, 2015).

Rhizobiophages are bacteriophages that infect rhizobium. A rhizosphere has a large number of bacteria around the root system thus a suitable place for phages (Swanson et al., 2009). This rhizobiophage for *Bradyrhizobium japonicum* decreases the nodulation process, foliar N, nitrogenase activity, and shoot weight (Ahmad and Morgan, 1994; Liu et al., 2019).

This study investigated how AMF could help *Vigna unguiculata* reduce the negative effects caused by rhizobiophage on nodulation and growth parameters.

Materials and Methods

Microorganisms

The Egyptian strain *Bradyrhizobium* sp. *Vigna (tal16)* was obtained as a ready-pure isolate and serologically characterized by Agriculture Research Center in Kafr El-Sheikh governorate, Egypt.

Phage VB_BrV_SD4 was isolated from *Vigna unguiculata* rhizosphere soils in the Met El-Ez region, Dakalia Governorate, Egypt. The single plaques of this phage were isolated according to procedures used by Dhar et al. (1979).

The vascular arbuscular mycorrhizae which included a mixture of *Funneliformis mosseae* (Walker &

Schubler Comb nov.) and *Rhizoglomus clarum* (Nicolson & Schenck) Sieverd., G.A. Silva & Oehl comb. Nov.) were obtained as a ready pure isolate from Mansoura, Dakhalia, Egypt.

Preparation of AMF inoculum

The inoculum of AMF species, including *Funneliformis mosseae* and *Rhizoglomus clarum*, were isolated from field soil cultivated by cowpea of Dakhalia Governorate, Egypt, using the wet sieving and decanting technique (Gerdemann and Nicolson, 1963) and identified by the author. The AMF spores identified were left to multiply for five months on onion (*Allium cepa*) plants. A mixture of plant roots and sand soil that contained the spores was used as the mycorrhizal inoculum.

Transmission electron microscope (TEM)

The morphological characteristics of phages were determined by TEM supported by the carbon-coated formvar film. The phages were negatively stained with 2 % (w v^{-1}) of the aqueous phosphate tungsten acid, pH 7.2 for 1 min. Then, air-dried for 1 h at room temperature in the Electron Microscope Unit, Mansoura, Dakhalia, Egypt according to procedures used by Ackermann and Prangishvili (2012).

Pot experiments

The pot experiment allows investigating combinations between mycorrhizae, Bradyrhizobium, and rhizobiophage in the growth and nodulation process. The experiment was conducted in plastic pots (25 × 30 cm: length × diameter) in controlled environmental greenhouse conditions (30 °C day/25 °C night temperatures, 70 % relative humidity, 16/8 h light/dark period cycle with a photosynthetic photon flux density of 500-700 mole m^{-2} s^{-1}) at Mansoura, Dakhalia (31°02′31.1″ N 31°21′10.0″ E, altitude 15 m). The soil used in the experiment was composed of clay: silt: sand (4:2:1 v v^{-1}) with 8.1 pH, 3.45 EC, 1.24 % OM, 65 mg kg^{-1} N, 10.5 mg kg^{-1} P, 124 mg kg^{-1} K. Each pot contained 6 kg of soil. Total P was extracted by nitric perchloric acid digestion and measured using the Vanado molybdo phosphoric acid colorimetric method (Jackson, 1973).

Total potassium (K) was assayed using a flame spectrophotometer. Total N was determined by the Kjeldahl method (Nelson and Sommers, 1973).

The research experiment included six treatments: (1) control, (2) VAM inoculation, (3) bradyrhizobium inoculation, (4) bradyrhizobium and VAM inoculation, (5) bradyrhizobium and rhizobiophage inoculation, and (6) bradyrhizobium, VAM and rhizobiophage inoculation. Every pot contained five cowpea seeds, which were thinned to thee after seven days of sowing. The trial was designed with three replications.

Bradyrhizobium cultivation

The germinated seedlings in sterile agar media were immersed for 1 h in a 3-day-old bradyrhizobium culture in yeast mannitol broth media containing 10^8 CFU mL^{-1} provided by sterilized Arabic gum solution (Heffner et al., 2009). Before sowing, a thin layer of mycorrhizal inoculum (15 gm mixed inoculums) was placed on the seeds at a depth of 4 cm. Non-mycorrhizal pots were given equal sterilized mycorrhizal inoculum to provide the same nutrient without mycorrhizal spores.

Phage VB_BrV_SD4 inoculation

In the phage treatment, phage VB_BrV_SD4 was mixed with the soil by mix 1.0 × 10^7 PGU g^{-1} soil (Hashem and Angle, 1990).

Fresh and Dry Weights

Fresh and dry weights were determined in cowpea shoots after 45 d of cultivating plants and the roots were carefully washed with tap water to minimize soil particles. Upon reaching a constant weight, the shoot and root samples reached were dried in an oven at 70 °C for 72 h. Dried samples were weighed on a scale of ± 0.001 g (Huang et al., 2017).

Chlorophylls a & b

The photosynthetic pigments, such as chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll, were determined by Metzner et al. (1965) and Horváth et al. (1972).

Leaf weight Chl.a = $(10.3\ A_{663} - 0.918\ A_{644})$

Weight Chl.b = $(19.7\ A_{644} - 3.870\ A_{663})$

Carotenoids = $4.2\ A_{452.5} - (0.0264$ Chl.a + 0.426 Chl.b).

Protein Content

The modified Lowry protein was measured as previously described by Hartee (1972).

N, P, and K contents

The contents of N, P and K were determined at the Kafr Elsheikh Governorate, according to procedures used by Chapman (1961).

Levels of mycorrhizal colonization

The mycorrhizal inoculation levels were determined in cowpea root tissues after clearing and staining in 0.05 % trypan blue in lactophenol (Phillips and Hayman, 1970).

Nitrogenase enzyme activity

The nitrogenase enzyme activity of root nodules was determined by acetylene reduction activity according to procedures used by Hardy et al. (1973).

Statistical analyses

The results of the present experiment are expressed as the mean of three replicates ± standard error. All findings were analyzed by SPSS software (version 15) (Levesque, 2007). Data were statistically analyzed using a one-way analysis of variance (ANOVA) with XLSTAT 2018 statistical software and the means were compared using the Newman-Keuls test (p > 5 %).

Results

Transmission electron microscope (TEM (of isolated phage (VB_BrV_SD4)

TEM showed the morphology of the selected phages. Phage VB_BrV_SD4 was isolated against *Bradyrhizobium* sp. *Vigna (tal169)* in Figure 1. It showed only a head and tail and belonged to the family phages (*Siphoviridae)*. The head shape was icosahedral in shape, measuring 43.44 nm. The long non-contractile tail was measured at 99.85 nm.

Nodule numbers, Nitrogenase enzyme activity, and dry weight

Cowpea plants (*Vigna unguiculata*) grown in soil containing phage VB_BrV_SD4 inoculated with *Bradyrhizobium* sp. *Vigna (tal16)* showed a significant reduction in nodulation where the nodule number reduced in the presence of phages from 62.67 to 35.33 (n per plant), nitrogenase from 3.70 to 2.20 (µmol C_2H_4 per plant h^{-1}), and dry weight of nodules from 0.07 to 0.034 (g per plant). However, mycorrhizal inoculation reduced these parameters from 44, 40.5, and 41.5 % to 20, 28, and 12 %, respectively (Table 1).

Mycorrhization frequency and intensity evaluation

Table 1 shows that the frequency and intensity of VAM of the cowpea plant. Although phage VB_BrV_SD4 significantly reduced mycorrhizal colonization levels, bradyrhizobium significantly increased them. Figure 2 shows the difference between mycorrhized and non-mycorrhized cowpea plant-stained roots.

Pigment content

Data in Table 2 show that the presence of phage VB_BrV_SD4 affected the total chlorophyll content and carotenoids, reducing the amount of total chlorophyll in the cowpea plant inoculated with bradyrhizobium from 19.55 to 17.88 and carotenoids from 8.68 to 7.08. However, mycorrhizal inoculation reduced total chlorophyll and carotenoids from 8.6 and 10 % to 6.4 and 4.8 %, respectively.

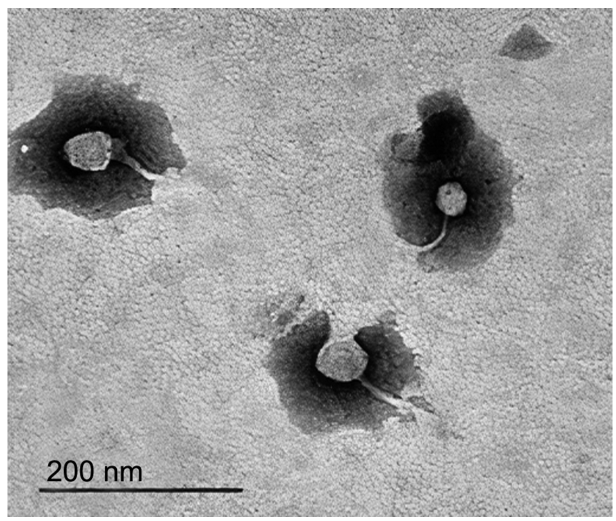

Figure 1 – Electron micrograph of rhizobiophage specific against Bradyrhizobium sp. Vigna (tal169).

Table 1 – Effect of bradyrhizobial, mycorrhizal, rhizobiophage inoculation in nodulation process, nitrogenase, and mycorrhizal inoculation in flowering stage.

Treatment	Number of nodules	Dry weight of nodules	Nitrogenase	Mycorrhizal inoculation level		
				F%	M%	A%
	n per plant	g per plant	µmol C_2H_4 per plant h^{-1}			
Control	9.67 ± 1.53[e]	0.01 ± 0.00[e]	0.00 ± 0.00[d]	0	0	0
M	12.00 ± 2.00[e]	0.01 ± 0.00[e]	0.00 ± 0.00[d]	15	27	63
R	62.67 ± 8.74[c]	0.07 ± 0.01[c]	3.70 ± 0.47[b]	0	0	0
R + M	118.00 ± 6.56[a]	0.125 ± 0.01[a]	5.30 ± 0.47[a]	17	31	100
R + Ph	35.33 ± 4.51[d]	0.034 ± 0.01[d]	2.20 ± 0.31[c]	0	0	0
R + M + Ph	94.33 ± 4.04[b]	0.11 ± 0.01[b]	3.80 ± 0.31[b]	17	17.52	70

The number marked with different superscript letters in the same column show statistic difference at significant level. R = bradyrhizobium; Ph = Phage Control: plant only; M = mycorrhizae; F% = Frequency of root inoculation; M% = Intensity of cortical infection; A% = Arbuscule frequency in roots.

N, P, and K content

In cowpea plants inoculated with bradyrhizobium, the presence of phage VB_BrV_SD4 decreased the N percentage in the shoots from 2.47 to 1.45. However, mycorrhizal inoculation alleviated the presence of phage and improved the reduction from 41.3 to 24.5 % (Table 3). Compared to non-mycorrhized therapies, mycorrhizae increased the N percentage in seeds.

The P and K contents were greatly influenced by mycorrhizae, bradyrhizobium, and rhizobiophage applications (Table 4). On the other hand, the addition

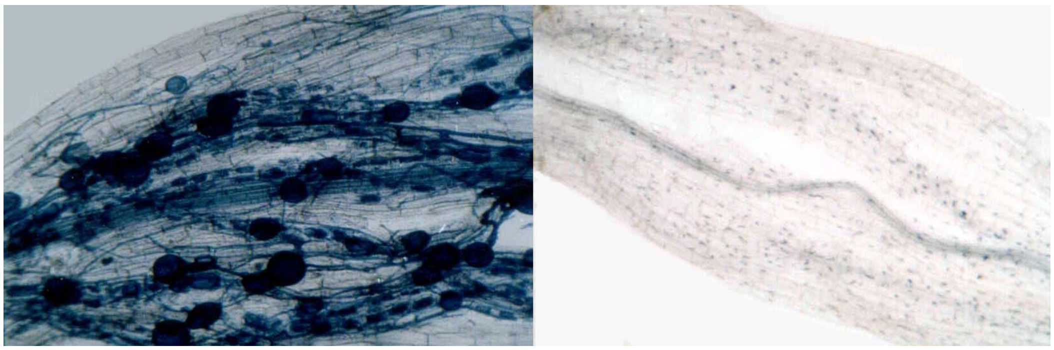

Figure 2 – Non–mycorrhizal segment (right) and mycorrhizal segment (left) of cowpea stained root.

Table 2 – Effect of bradyrhizobial, mycorrhizal, rhizobiophage inoculation in chlorophyll a, chlorophyll b and carotenoids in flowering stage.

Treatment	Total (a + b)	Carotenoid	Chlorophyll (b)	Chlorophyll (a)
Control	14.26 ± 0.84^{d}	6.38 ± 0.49^{c}	5.77 ± 0.29^{d}	8.49 ± 0.96^{d}
M	20.65 ± 0.37^{ab}	8.72 ± 1.01^{a}	7.87 ± 0.39^{b}	12.78 ± 0.74^{ab}
R	19.55 ± 0.30^{b}	8.68 ± 1.17^{ab}	7.82 ± 0.17^{bc}	11.73 ± 0.47^{b}
R + M	22.00 ± 1.46^{a}	10.00 ± 1.01^{a}	8.69 ± 0.65^{a}	13.31 ± 0.88^{a}
R + Ph	17.88 ± 0.40^{c}	7.08 ± 0.58^{bc}	7.17 ± 0.27^{c}	10.17 ± 0.14^{c}
R + M + Ph	20.60 ± 1.44^{ab}	9.46 ± 0.99^{a}	7.87 ± 0.42^{b}	12.73 ± 1.33^{ab}

The number marked with different superscript letters in the same column show statistic difference at significant level. R = bradyrhizobium; Ph = Phage Control: plant only; M = mycorrhizae.

Table 3 – Effect of bradyrhizobial, mycorrhizal, rhizobiophage inoculation in shoot fresh weight, shoot dry weight, length of shoot, length of root and number of leaves in flowering stage.

Treatment	Shoot fresh weight	Shoot dry weight	Length of shoot	Length of root	Number of leaves
	g per plant		cm per plant		n per plant
Control	18.43 ±0.67^{e}	3.13 ± 0.23^{d}	30.00 ± 2.00^{c}	8.83 ± 1.04^{e}	19.67 ± 2.52^{b}
M	27.17 ± 0.15^{b}	5.13 ± 0.25^{b}	36.53 ± 1.35^{ab}	12.67 ± 1.15^{bc}	26.67 ± 6.51^{a}
R	23.70 ± 1.47^{c}	4.28 ± 0.38^{c}	33.00 ± 3.00^{bc}	11.27 ± 1.25^{cd}	23.67 ± 3.51^{ab}
R + M	30.73 ± 1.60^{a}	5.68 ± 0.24^{a}	42.43 ± 6.51^{a}	15.87 ± 1.03^{a}	29.67 ± 3.51^{a}
R + Ph	21.90 ± 0.56^{d}	3.77 ± 0.42^{c}	30.83 ± 2.57^{bc}	9.17 ± 1.61^{de}	23.00 ± 3.00^{ab}
R + M + Ph	28.73 ± 0.51^{b}	5.24 ± 0.14^{ab}	41.67 ± 2.14^{a}	14.13 ± 1.03^{ab}	28.67 ± 2.52^{a}

The number marked with different superscript letters in the same column show Statistic difference at significant level. R = bradyrhizobium; Ph = Phage Control: plant only; M = mycorrhizae.

Table 4 – Effect of bradyrhizobial, mycorrhizal, rhizobiophage inoculation in nitrogen, phosphorus, potassium in flowering and yield stage.

Treatment	Shoot system in flowering stage			Seeds in yield stage		
	N	P	K	N	P	K
	%		μg g^{-1}	%		μg g^{-1}
Control	0.51 ± 0.18^{c}	0.44 ± 0.08^{d}	132.00 ± 9.45^{c}	1.62 ± 0.78^{e}	0.46 ± 0.04^{d}	68.67 ± 3.01^{d}
M	1.03 ± 0.61^{c}	3.23 ± 0.31^{bc}	152.00 ± 3.64^{b}	3.11 ± 0.18^{bc}	3.47 ± 0.50^{bc}	84.17 ± 1.89^{b}
R	2.47 ± 0.91^{ab}	2.80 ± 0.30^{c}	148.66 ± 4.04^{b}	2.40 ± 0.62^{cd}	3.46 ± 0.50^{bc}	77.66 ± 3.01^{c}
R + M	2.99 ± 0.65^{a}	4.40 ± 0.36^{a}	193.00 ± 11.36^{a}	4.33 ± 0.38^{a}	5.04 ± 0.63^{a}	126.33 ± 5.13^{a}
R + Ph	1.45 ± 0.61^{bc}	2.67 ± 0.60^{c}	144.33 ± 5.1^{c}	2.30 ± 0.49^{de}	3.09 ± 0.32^{c}	76.50 ± 4.27^{c}
R + M + Ph	2.26 ± 0.51^{ab}	3.75 ± 0.41^{ab}	154.67 ± 5.86^{b}	3.74 ± 0.59^{ab}	4.19 ± 0.30^{b}	86.00 ± 1.32^{b}

The number marked with different superscript letters in the same column show statistic difference at significant level. R = bradyrhizobium; Ph = Phage Control: plant only; M = mycorrhizae.

of rhizobiophage significantly decreased these contents in the non-mycorrhized cowpea plant. Compared to cowpea plants infected with or without lytic phage, these contents in the dual inoculation of bradyrhizobium and mycorrhizae were significantly higher.

Growth and yield parameters

Table 4 shows that phage VB_BrV_SD4 reduced all growth measurements of the cowpea plant inoculated with bradyrhizobium from 6.6 to 18.6 %. However, mycorrhizal inoculation alleviated the presence of phage and reduced from 1.8 to 11 %.

According to Tables 5 and 6, the presence of phage VB_BrV_SD4 reduced all yield measurements of the cowpea plant inoculated with bradyrhizobium from 12 to 18.8 %. However, mycorrhizal inoculation alleviated the presence of phage and reduced from 10 to 4.8 %.

Protein content

Table 6 shows that phage VB_BrV_SD4 reduced the amount of protein in seeds in the cowpea plant inoculated with bradyrhizobium from 187.33 to 147.33, but mycorrhizal inoculation reduced the presence of phage from 21.4 to 11.8 %.

Discussion

Rhizobiophages influence soil rhizobium (Hashem and Angle, 1990; Liu et al., 2019). However, there is little information about alleviating the negative effect of phages by mycorrhizae, to the best our knowledge. Therefore, this study investigated the reduction of the negative effects of rhizobiophage on the growth and nodulation of the valuable crop *Vigna unguiculata*.

The isolated phage VB_BrV_SD4, which belongs to the *Siphoviridae* family, has an icosahedral head with a diameter of 43.44 nm and a long non-contractile tail, measuring 99.85 nm. Phage VB_BrV_SD4 is similar to phage Z that has an icosahedral head and a non-contractile tail, according to Jamal et al. (2015). It belongs to the family *Siphoviridae* of the order *Caudovirales.*

The results revealed an increase in nodule numbers, nodule dry weight, nitrogenase activity, and plant dry weight of *Vigna unguiculata* inoculated with bradyrhizobium sp. The presence of phage VB_BrV_SD4 reduced Vigna *(tal16)*. These findings corroborate results from previous studies (Liu et al., 2019).

Bradyrhizobial inoculation stimulated the nodulation process that affected growth, yield measurements, and chlorophyll and P contents. These results are in agreement with Arafa et al. (2018) and Verma et al. (2014), who reported that inoculation of cowpea and fenugreek seeds with effective bradyrhizobial strains significantly stimulated yield, total protein yield %, N and P uptake and total carbohydrate % in grains and seeds of the two plants.

VAM increased P absorption, affecting yield, growth, chlorophyll, protein, and N accumulation in the plant. These findings are consistent with Yaseen et al. (2016). The authors found that mycorrhizal inoculation increased plant yield and chlorophyll content on the leaves of many plants because P is the primary energy source for plants to absorb N and develop.

The dual inoculation treatment of VAM and bradyrhizobium in cowpea plants increased growth and yield measurements, chlorophyll, protein, and nodulation process at their maximum value compared to all treatments.

These results are similar to findings of previous studies, which demonstrated that the dual inoculation of seeds with mycorrhizae and bradyrhizobium promoted an increase in all measurements in the cowpea plant due to the increasing photosynthesis process (Moradi et al., 2013; Yaseen et al., 2016), possibly due to the presence

Table 5 – Effect of bradyrhizobial, mycorrhizal, rhizobiophage inoculation in length of shoot, length of root, length of legume in yield stage.

Treatment	Length of legume	Number of seeds
	cm per legume	n per legume
Control	10.83 ± 0.76[d]	6.00 ± 2.00[d]
M	13.83 ± 0.76[ab]	9.67 ± 0.58[bc]
R	12.83 ± 0.76[bc]	8.66 ± 1.15[c]
R + M	15.80 ± 1.04[a]	13.33 ± 0.58[a]
R + Ph	10.83 ± 0.76[d]	8.00 ± 1.00[cd]
R + M + Ph	14.67 ± 1.15[ab]	11.33 ± 1.53[ab]

The number marked with different superscript letters in the same column show statistic difference at significant level. R = bradyrhizobium; Ph = Phage Control: plant only; M = mycorrhizae.

Table 6 – Effect of bradyrhizobial, mycorrhizal, rhizobiophage inoculation in number of seeds, number of legumes, weight of 100 seeds, shoot fresh weight, shoot dry weight and protein in seeds in yield stage.

Treatment	Number of legumes	Weight of 100 seeds	Shoot dry weight	Protein
	n per plant	g per plant	g per plant	mg per g fresh weight
Control	16.67 ± 1.15[e]	16.27 ± 0.54[f]	12.33 ± 0.55[e]	127.33 ± 15.56[e]
M	30.67 ± 2.08[b]	22.13 ± 0.67[c]	21.60 ± 0.62[c]	211.33 ± 24.00b[c]
R	26.66 ± 1.53[c]	20.25 ± 0.67[d]	18.54 ± 0.57[d]	187.33 ± 10.50[cd]
R + M	36.33 ± 1.53[a]	26.30 ± 0.49[a]	26.71 ± 0.97[a]	270.33 ± 39.72[a]
R + Ph	21.66 ± 1.53[d]	18.78 ± 0.51[e]	17.37 ± 0.57[d]	147.33 ± 6.81[de]
R + M + Ph	32.33 ± 1.53[b]	24.09 ± 1.22[b]	23.63 ± 1.43[b]	238.67 ± 38.21[ab]

The number marked with different superscript letters in the same column show statistic difference at significant level. R = bradyrhizobium; Ph = Phage Control: plant only; M = mycorrhizae.

of a high amount of protein and P accumulation by bradyrhizobium and mycorrhizae. In the dual inoculation procedure, the mycorrhizal colonization and P content were at the maximum value. These findings seem to be consistent with other studies that reported a greater effect on nodulation, mycorrhizal colonization, and P content than mono-inoculation because of the dual inoculation (Stancheva et al., 2006).

The results show that the nutrients absorbed by bradyrhizobium and mycorrhizae to the plants were at a maximum value in the dual inoculation, supporting previous studies (Abdel-Fattah et al., 2016; Abdullahi and Sheriff, 2013; Khalil and Yousef, 2014). The hyphae took the nutrients to plants, leading to efficient mobilization and uptake of P, N, and P transported to the plant.

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Authors' Contributions

Conceptualization: El-Didamony, G.; Allam, Y.; Amin, G.; Abdel-Fattah, G**. Data acquisition**: Egamberdieva, D.; Abd_Allah, E.F. **Data analysis:** Allam, Y.; Amin, G.; Abdel-Fattah, G. **Design of methodology:** El-Didamony, G.; Allam, Y.; Amin, G.; Abdel-Fattah, G. **Writing and editing:** Egamberdieva, D.; Abd_Allah, E.F.; Hashem, A.

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