DOI: 10.22620/agrisci.2021.31.014

EFFECT OF FIVE *BACILLUS* STRAINS ON SEED GERMINATION OF *PISUM SATIVUM* AND PLANT GROWTH OF ORIGANUM VULGARE SUBSP. HIRTUM (OREGANO)

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Abstract

This study aims to investigate the effect of PGP-strains from genus *Bacillus* on seed germination of *Pisum sativum* and plant growth of *Origanum vulgare* subsp. *hirtum* in comparison with two growth plant regulators.

Bacterial cell free supernatants (CFS) from five *Bacillus* strains were used in two different concentrations – 100 and 500 fold dilutions. The growth regulators (gibberellic acid and indole acetic acid) were used to determine optimal concentration to *Pisum sativum* seeds germination and plant growth, and were compared with the bacterial CFS.

PGP-activities of CFS and plant regulators were evaluated by morphometric data of sprouted seeds and plants. Bacterial CFS increase the number of lateral root branching and the average number of leaves compared with the tested growth regulators.

The growth regulators had positive effect on plant growth, although their accumulation in the soils has been shown to cause soil erosion and pollution. The treatment of *Origanum vulgare* subsp. *hirtum* with bacterial CS from *B. subtilis* 8VR, *B. pumilus* 9VR and *B. thuringiensis* 13VR, resulted in significant increase in the root length compared to the control. The maximum length of the main root was measured after treatment with CS from *B. thuringiensis* 13VR. Bacterial CS from genus *Bacillus* had no effect of stem length of *Origanum vulgare* subsp. *hirtum*, while CS from *B. cereus* 7VR and *B. subtilis* 8VR increased the number of leaf trichomes compared to the control.

Key words: *Bacillus*, seed germination, *Pisum sativum*, *Origanum vulgare* subsp. *hirtum*, growth regulators.

INTRODUCTION

Pea (*Pisum sativum* L.) is recognized as an important source of protein for human populations around the world. Some bacteria are associated with roots of crop plants and exert beneficial effects on their host and are referred to as plant growth promoting bacteria (PGPR). PGPR are free living bacteria that may impart beneficial effects on plants. PGPR colonize roots and stimulate overall plant growth. They also improve seed germination, root development, mineral nutrition and water utilization (Nelson et al., 2004; Osman N. I. et al. 2018). Among rhizospheric microbial community, members of the genus *Bacillus* are the most abundant soil resident that possess several PGP traits. They use several mechanisms to improve and enhance plant growth and its productivity by both direct and indirect mechanisms under various environmental conditions. They facilitate plant growth by producing phytohormones, exo-polysaccharide (EPS), hydrogen cyanide (HCN), lytic enzymes, antibiosis, induced systemic resistance (ISR) (Akinrinlola R. J. at al., 2018; Singh I., 2018: Tiwari S. et. al., 2019).

MATERIALS AND METHODS

Bacterial strains: Five rhizospheric strains from genus *Bacillus*: *Bacillus subtilis* 6VR, *Bacillus cereus* 7VR, *Bacillus subtilis* 8VR, *Bacillus pumilus* 9VR and *Bacillus thuringiensis* 13VR were used.

Plants and seeds: In the first experiment we used *Pisum sativum* seeds (Peas), Velida 1 LT, Plovdiv, Bulgaria; and in the second experiment – *Origanum vulgare* subsp. *hirtum*were plants.

Growth regulators: We used Indole acetic acid (IAA) and Gibberellic acid 3 (GA_3), Duchefa Biochemics, Netherlands.

Growth of bacterial strains: Five bacterial strains were grown in 100 mL *14'* media at 250-mL Erlenmeyer flask on a shaker (250 rpm) at 28 ºC for 120 hour batch fermentation process. The cell supernatant (CS) obtained from each strain was centrifuged at 7500 g for 15 minutes to produce cell free supernatant (CFS) of each strain. The working CFS concentrations were prepared by diluting each CFS 100x and 500x with distilled water.

Experiment I

In the first part of the *in vitro* experiment, seeds of *Pisum sativum* were treated with both working concentrations of CFS obtained from the *Bacillus* strains. The germination and development of the pea seeds were monitored in a model phytochamber under controlled temperature and light conditions. The control seeds were treated with water and incubated in the same manner. At the end of the experiment, several morphometric parameters were measured: length of main root, length of stem, number of branches on the main root, number of pear leaves. The same parameters were measured in the control.

In the second part of the experiment, the effects of the growth regulators on the pea seeds germination and development were tracked. Five different concentrations (0,3 µg, 0,6 µg, 0,9 µg, 1,2 µg and 1,5 µg) of commercial growth regulators (GA_3 and IAA) were used to determine their effect in comparison to a control treatment with water. At the end of the experiment, the already mentioned morphometric parameters were measured.

Experiment II

In vivo pot experiment with *Origano vulgare* subsp. *hirtum.* At the first part of the experiment, the effect of cell supernatants (CS) from the studied bacterial strains on oregano plants was studied. The experimental layout consisted of 21 pots filled with sterilized send - peat mixture from *Teramix (*Agro CS LTD, Czech Republic). Each pot was planted with one oregano seedling. The plants were divided into 6 experimental groups, one per bacillus strain, and irrigated with 200 ml of dissolved CS ~ 10^7 cfu/ml (2 ml/l).

In the second part of the *in vivo* experiment, oregano plants were treated with the commercial growth regulators (IAA and GA_3). Three different concentration of commercial growth regulators (600µg IAA/250 ml H_2O, 600µg GA_3/250 ml H_2O, 300µg IAA and GA_3 at ratio 1:1) were used to investigate their effects on plant growth. After plant harvest, several morphometric parameters were measured: length of main root, length of stem and the number of trichomes/cm². The same parameters were measured in the control.

Statistical analysis

Unidirectional analysis of variance (ANOVA) was performed on each variable, and when differences were significant, the LSD (least significant difference) test was performed.

RESULTS AND DISCUSSION

At the first experiment we conducted comparative study on seed germination after treatment of *Pisum sativum* seeds with CFS of *Bacillus* strains and commercial growth regulators (IAA and GA_3). Application of *Bacillus* CFSs had negative effect to length of the root and stem of germinating pea seeds (fig.1a and fig.2 a) (Osman N. I. et al, 2018). The inhibition of seminal root growth by the

Bacillus spp. treatments can be imputed to auxin induced synthesis of ethylene which inhibits root elongation at high concentrations. Indole acetic acid synthesized by bacteria may be involved in the rise of ethylene level to an excessive concentration as demonstrated by (Romano et al., 1993,). Various experiments have shown that endophytic *Bacillus* spp. greatly increase their auxin production (Araújo et al., 2005; Murugappan et al., 2013; Talboys et al., 2014). Despite the reduced elongation of the root, the treatment with bacterial CFS increased the root branching (fig.4. a) and the number of pear leaves (fig.5 a) compared to the control. The highest number of branches (32) was detected under treatment with 100x diluted CFS derived from *Bacillus subtilis* 8VR. Inoculation of the seeds of garden pea with CFS increased the number of lateral branching of the main root compared to the control, due to the production of auxins. We can expect that the enhanced lateral root formation increases the nutrients uptake capacity of the plants.

Application of 0,3μg GA_3 and 1,5μg GA_3 lead to slightly increased length of the roots, compared to the control (fig.1.b). The treatment of the seeds with 0,3μg, 0,6μg, 0,9μg, 1,2μg and 1,5μg GA_3 leads to significant increase in stem length compared to the control (fig. 2. b). There is a tendency toward reduction of the root and stem length of germinating seeds by increasing concentrations of IAA (fig.1. b). Slight increase of stem length was detected only in the treatment with 0,3μg IAA (fig.3. b). Treatment of the seeds with 0,3μg, 0,9μg and 1,2μg GA_3 decreased the number of root branches compared to the control (fig.3. b). Only application of 0,3μg and 0,6μg IAA increases the number of root branches (fig.3 b). In contrast of auxins, studies on the effects of GA on roots are limited, though there are many studies on their effects on aerial organs (Man˜eroa F. J.G., 2001; Tanimoto et. al., 2005; Pandya N. D. et al., 2013).

Applications of CFS obtain from *Bacillus subtilis* 6VR (100x and 500x), *Bacillus cereus* 7VR (100x and 500x), *Bacillus subtilis* 8VR (500x) and *Bacillus pumilus* 9VR (500x) increased the number of leaves of sprouting seeds compared to the control (fig.4 a). The highest number of leaves (4 pears) was observed after applying CFS derived from *B. cereus* 7 VR (100x). The application of *Bacillus thuringiensis* 13VR (100x and 500x) CFSs decreased the number of leaves compared to the control. The effect of the growth regulators on the number of leaves is less pronounced in comparison to the CFSs.

At the second *in vivo* experiment we evaluated the effects CS derived from *Bacillus* spp. and growth regulators on plant growth of *Origanum vulgare* subs. *hirtum* (tab.3). Plants treated with CS showed significant increase of root length compared to the control (fig. 5 a). The highest average length of the roots was observed in plants treated with CS from strain *Bacillus thuringiensis* 13VR. The smallest average length of the root was observed after application of CS from *B. subtilis* 6VR. Application of the two growth regulators did not stimulate the root development compared to the control. A slight increase of the average root length was detected, only in the treatment with GA_3 (fig.5b). Combined treatment with both growth regulators ($IAA+GA_3$) also decreased the root length compared to the control. We can conclude that application of CS leads to significant increase of the average root length compared to treatment with growth regulators.

In general, the application of CS leads to a decrease in average stem length of plants. The only increase of stem length, compared to the control, was observed in the treatment with CS of *Bacillus pumilus* 9VR (fig.6.a). The strongest effect on stem length of oregano plants was detected after treatment of the plants with combination of the growth regulators (fig.6.b). Contrary, a decreased stem length of the plant was observed in the separate application of the growth regulators.

We have studied the effects of CS on the number of leaf trichomes of the treated Oregano plants (fig.7.a). An increase in the number of trichomes was observed in the treatment with CS from *B. subtilis* 8VR and *B. cereus* 7VR. The highest average number – 58 trichoms/cm² was detected under treatment with CS from *Bacillus subtilis* 8VR. Plants treated with IAA and combination of growth regulators showed increased number of trichomes compared to the control. Application of gibberellinic acid had no effect on the number of trichomes.

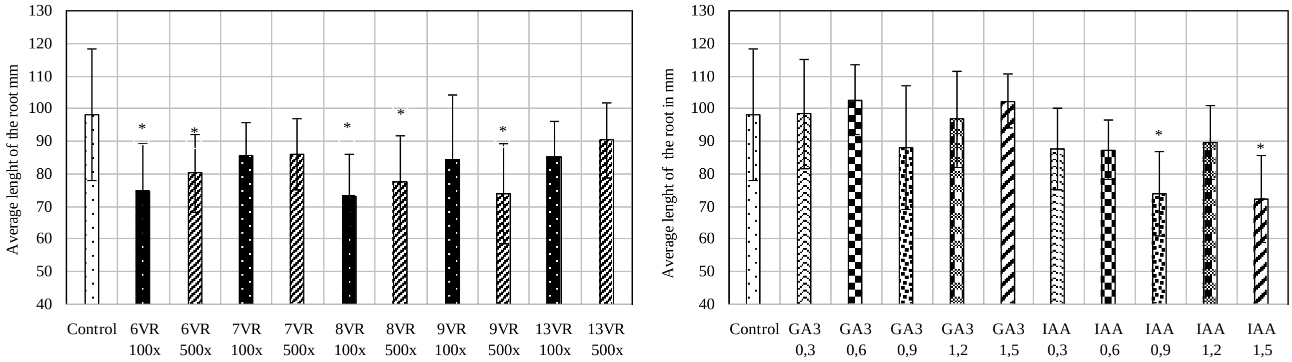

Fig. 1. Length of the main root of *Pisum sativum* seeds after treatment with: CFSs (a); commercial growth regulators (IAA and GA_3) (b) *$p \leq 0.05$.

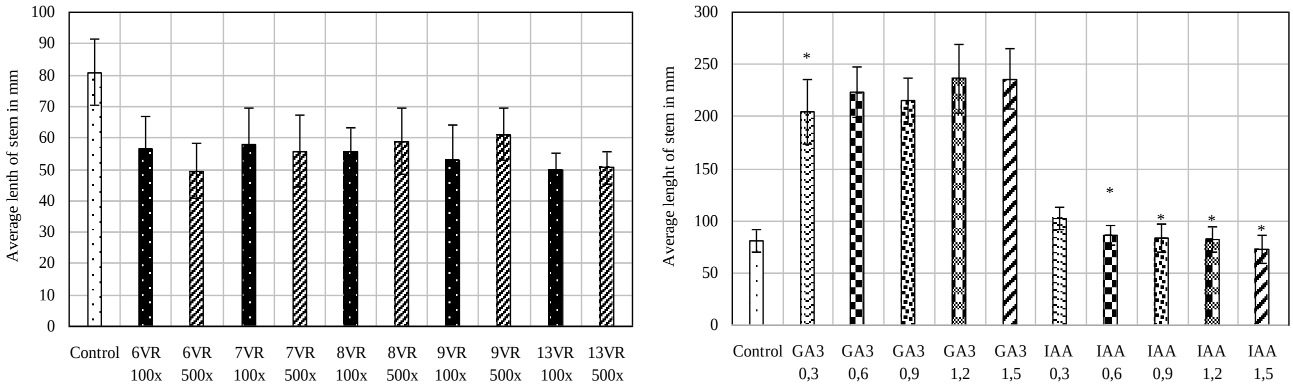

Fig.2. Length of *Pisum sativum* stem after treatment with: CFS (a); commercial growth regulators (IAA and GA_3) (b) *$p \leq 0.05$

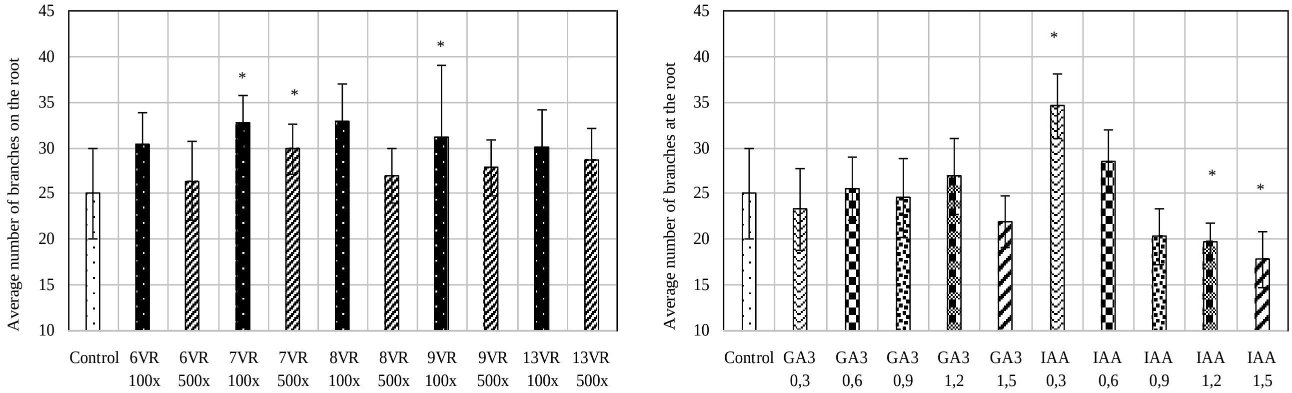

Fig. 3. Number of branches on the *Pisum sativum* root after treatment with: CFS (a); commercial growth regulators (IAA and GA_3) (b) *$p \leq 0.05$

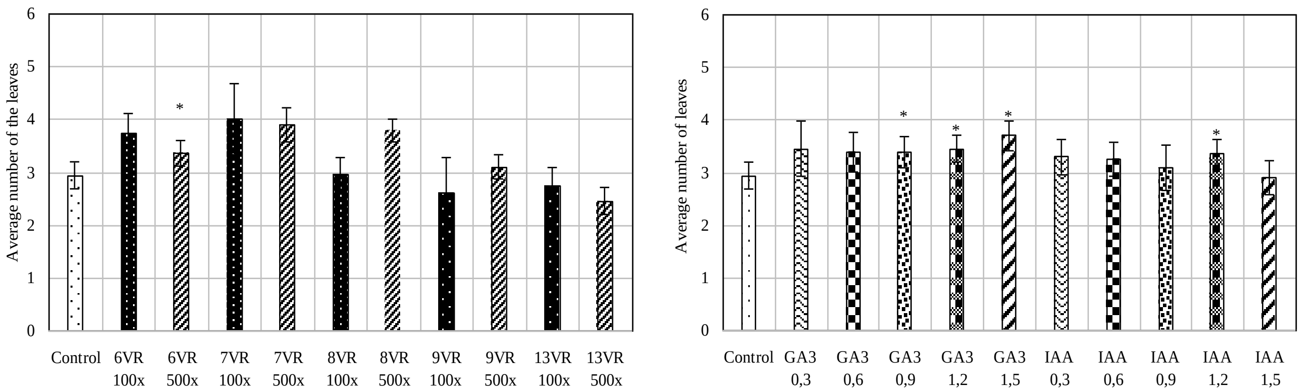

Fig. 4. Average number of leaves at seeds of *Pisum sativum* (garden peas) after treatment with: CFS (a); commercial growth regulators (IAA and GA_3) (b) $*p \leq 0.05$

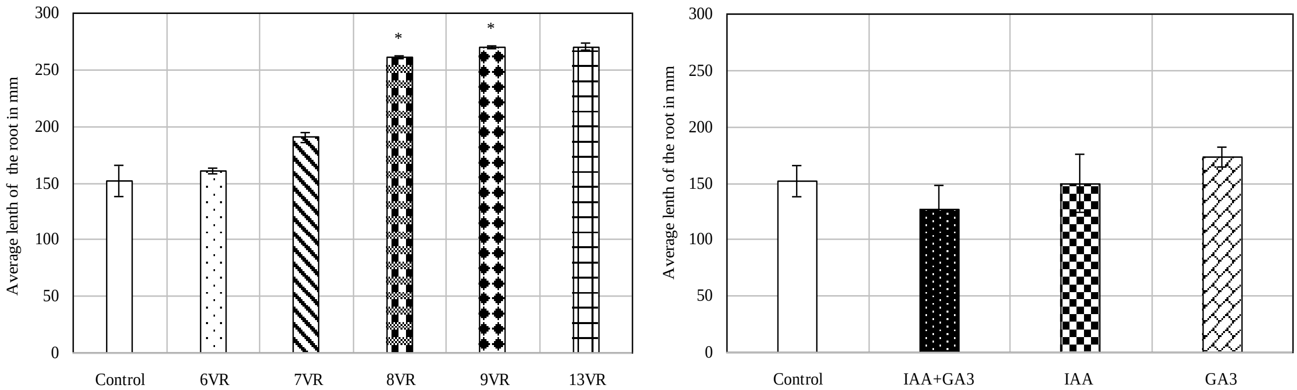

Fig. 5. Average length of the root of *Origanum vulgare* subsp. *hirtum* after treatment with: CS (a); commercial growth regulators (IAA and GA_3) (b) $*p \leq 0.05$

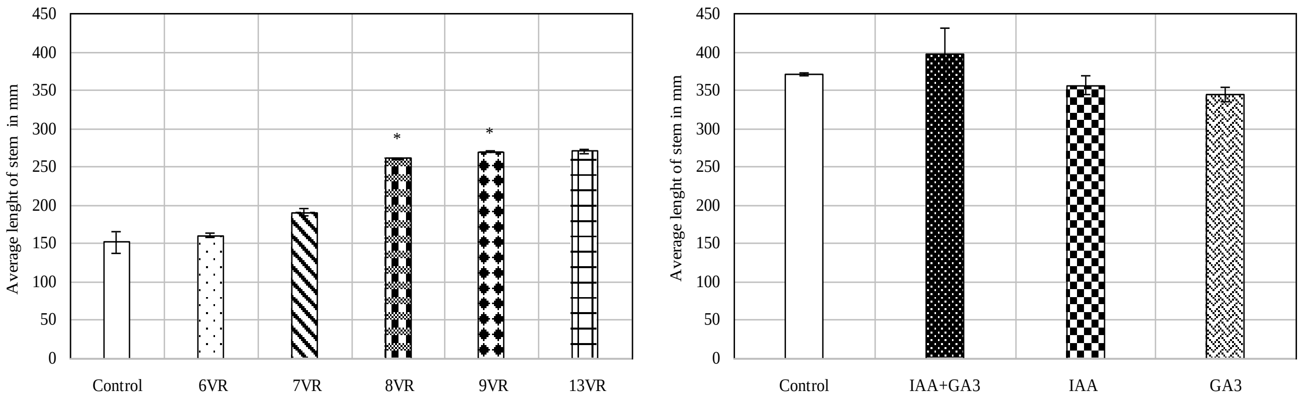

Fig. 6. Average length at stem to *Origanum vulgare* subsp. *hirtum* after treatment with: CS (a); commercial growth regulators (IAA and GA_3) (b) $* p \leq 0.05$

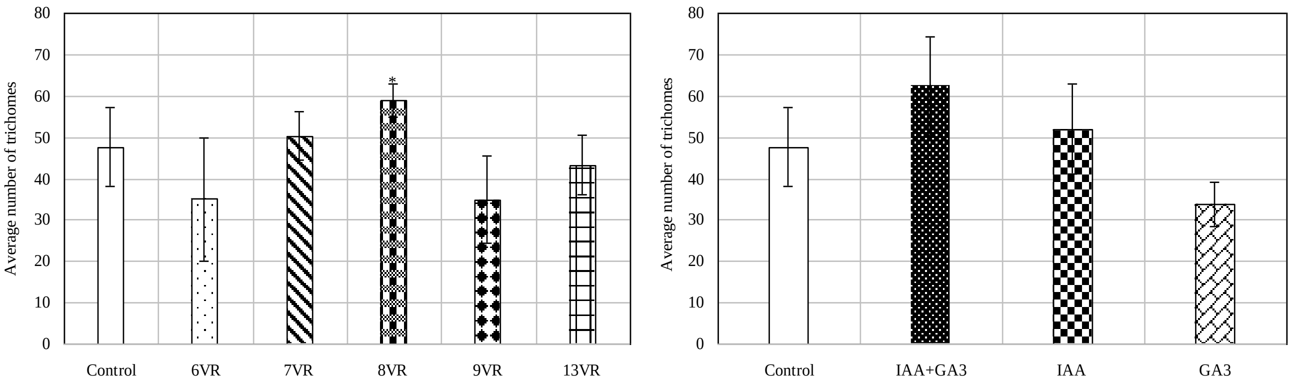

Fig.7. Average number of trichomes to plants of *Origanum vulgare* subsp. *hirtum* after treatment with: CS (a); commercial growth regulators (IAA and GA_3) (b) *$p \leq 0.05$

CONCLUSION

The studied *Bacillus* strains promote *in vitro* seed germination of *Pisum sativum* as it is evident by the increased number of branches of the main root and number of sprout leaves compared to the growth regulators and the control. *Bacillus* strains had no effect on root and stem lengths of *Pisum sativum* seedlings. Among used growth regulators only GA was shown to promote stem length.

In the *in vivo* assay CSs from *Bacillus* strains 8VR and 9VR showed highest PGP activity by promoting root elongation, average stem length and the number of trichomes in *Origanum vulgare* subsp. *hirtum,* compared to the control and the growth regulators. Treatment of the plants with growth regulators resulted in a slight, but not significant increase of root and stem length compared to the control.

The results of our study show the potential of different *Bacillus* spp. applications as an alternative to chemical fertilizer. Amongst the advantages of CFS and CS are: (1) highly effective in low dosages; (2) reduced potential of soil pollution; (3) lower price in comparison to the commercial growth regulators, and (4) ease of application. However, further investigations are needed to assess the PGP activity of *Bacillus* strains as biofertilizer in field conditions.

ACKNOWLEGMENTS

This work was supported by the Bulgarian Ministry of Education and Science under the National Research Programme “Healthy Foods for a Strong Bio-Economy and Quality of Life” approved by DCM # 577 /17.08.2018.

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