PRODUCTION OF SEEDS CONTAINING ENDOPHITIC BACTERIA IN SOME VEGETABLE SPECIES AND DETERMINING THE DEVELOPMENT AND BIOMASS DIFFERENCES OF PLANTS GROWN FROM THESE SEEDS

Dr. Kutay Coşkun YILDIRIM [a]

Assoc. Dr. Didem CANİK OREL [b]

Hilal OKYAY [c]

Mükerrem Melis GÜRSAN [a]

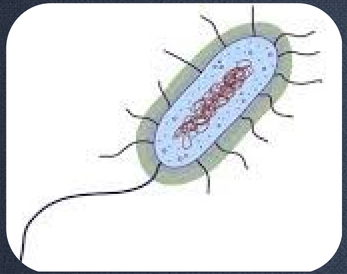
Günay YILDIZ [a]

a: Atatürk Horticultural Central Research Institute – Yalova / TÜRKİYE
b: University of Ankara, Faculty of Agriculture, Department of Plant Protection – Ankara / TÜRKİYE
c: University of Sütçü Imam, Faculty of Agriculture, Department of Horticulture – K.Maraş / TÜRKİYE

Presentation Plan

ABSTRACT

- In this research, it was aimed to transfer endophytic bacteria into some vegetable seeds and examining possible growth and biomass differences in plants produced from these seeds.



Pseudomonas spp.
Bacillus spp.

Tomato (Şencan 9 cultivar)

Eggplant (Pala 49 cultivar)

- A comparison of seeds containing endophytic bacteria and seeds inoculated with bacteria on the seed coat was also made.

1 . INTRODUCTION

TAGEM R&D AND INNOVATION

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

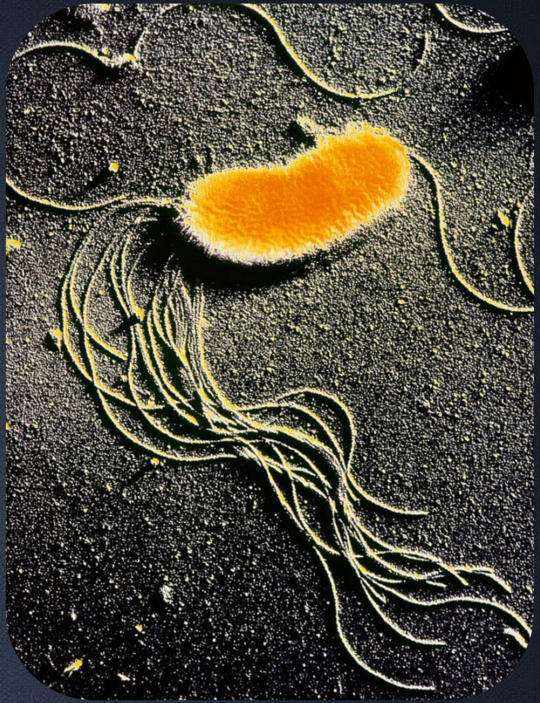
- Conservation of natural resources,
  -re-establishment of the deteriorated ecological balance,
  -keeping the soil alive,
  -protection of flora and fauna,
  - continuation of biodiversity,
  -destruction of toxic residues and chemical pollution

has been the main goals of humanity (Kotan 2002, Çakmakçı and Erdoğan 2005).

- All plants in nature are associated with many microorganisms, and these microorganisms play an important role in the growth and development of the plant. Endophytic bacteria, which are among these microorganisms, are bacteria that have been found to have many benefits, living in the tissues of plants, providing nutrients to the plant and/or colonizing these tissues without harming the plant (Farrar et al. 2014).

- Studies have shown that these bacteria have many benefits for the host plant.

- ❑ Increasing the growth rate,
- ❑ Higher biomass yield,
- ❑ Additional defense mechanism against pathogens,
- ❑ Biological nitrogen fixation,
- ❑ Separation of phosphate and iron,
- ❑ Production of indole acetic acid, cytokinin, gibberellin,
- ❑ Reducing ethylene synthesis and the effect of stress factors



Pseudomonas fluorescens

Gardner et al. 1982, Kempe and Sequeria 1983, Bashan 1986, Lalande et al. 1989, Misaghi and Donndelinger 1990, Chen et al. 1994, 1995, Musson et al. 1995, Shishido et al. 1996, Sturz et al. 1997, James 2000, Lee et al. 2004, Rosenblueth and Martinez-Romero 2006, Sziderics et al. 2007, Weyens et al. 2009, Compant et al. 2010, Glick 2012, Luo et al. 2012, Rashid et al. 2012, Mitter et al. 2013, Narula et al. 2013, Kotan 2014, Pandya et al. 2015, Coutinho et al. 2015, Saini et al. 2015

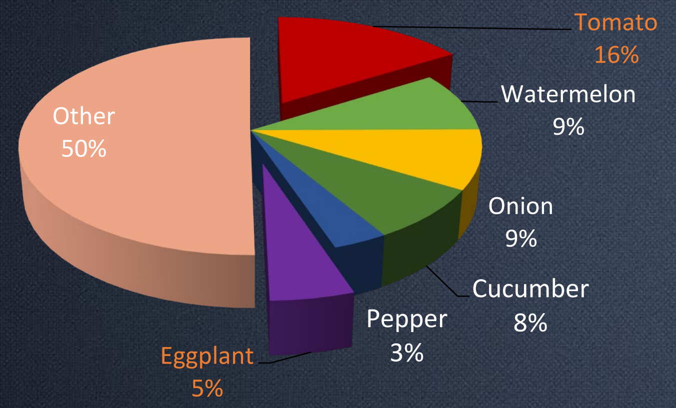
❖Why did we choose Tomato and Eggplant ??

- ❖In the comprehensive literature review of this study, only one article was found;
- ❖In the article published by Mitter et al. (2017), they successfully transferred *Paraburkholderia phytofirmans* bacteria into pepper seeds through stigma. In this respect, due to the similarity in flower structures, tomato and eggplant plants belonging to the Solanaceae family were chosen.

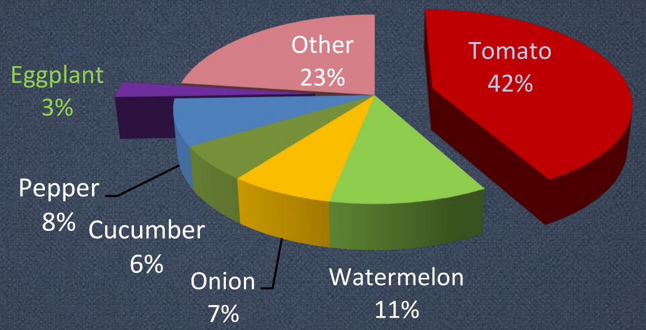
REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

❑ Production quantities and rankings of TOMATO and EGGPLANT species in the world and in Türkiye

World Vegetable Production 2019 FAO



Vegetable Production of Türkiye 2021 TÜİK



World Vegetable Production 2019 FAO

Rank	Species	Production (tonne)
1	Tomato	180.770.000
2	Watermelon	100.414.933
3	Onion	99.970.000
4	Cucumber	87.810.000
5	Eggplant	55.200.000
6	Pepper	38.030.000
7	Other	568.504.067

Vegetable Production of Türkiye 2021 TÜİK

Rank	Species	Production (tonne)
1	Tomato	13.204.015
2	Watermelon	3.491.554
3	Onion	2.280.000
4	Pepper	**2.519.938**
5	Cucumber	**1.886.239**
6	Eggplant	835.422
7	Other	7.194.120

❖ Türkiye ranks 6th in the world in tomato production and 4th in eggplant production after China (FAO, 2019).

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

2 . Purpose of the study and literature summary

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

Objectives of the study

- Currently used inoculation methods are not sufficient for endophytic bacteria to colonize the plant at the desired level. In addition, the industrial application of these methods is difficult because it increases the cost and time requirement of seed producers.

- Although it was determined that the seed coating method increased the germination and seedling growth of the seeds and the resistance against stress factors, it was observed that the bacteria generally did not survive at the desired level or could not colonize the plant sufficiently.

- It is believed that the reason for this situation is that the bacteria remain outside the seed coat and cannot enter the seed.

Objectives of the study

1- Transfer of endophytic bacteria to the stigma by spraying and colonization of the bacteria in the seeds at the stage of development

2- Detection of colonized bacteria in seeds

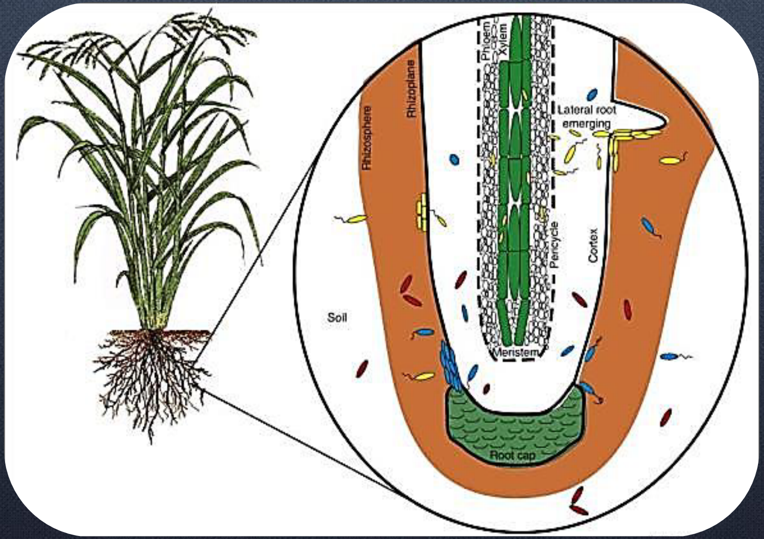
3- Identification of endophytic bacteria transferred into seedlings and determination of their amount

4- Examination of the seeds containing endophytic bacteria in terms of plant growth and comparison with seed coating method

Previous studies

- Endophytic bacteria can give more effective results than chemical applications by activating systemic resistance, especially when applied to healthy plants (Kuc 2001).

- The mechanisms of endophytic bacteria have not been fully elucidated on the plant and studies continue with increasing interest in terms of the advantages they provide to the plant.

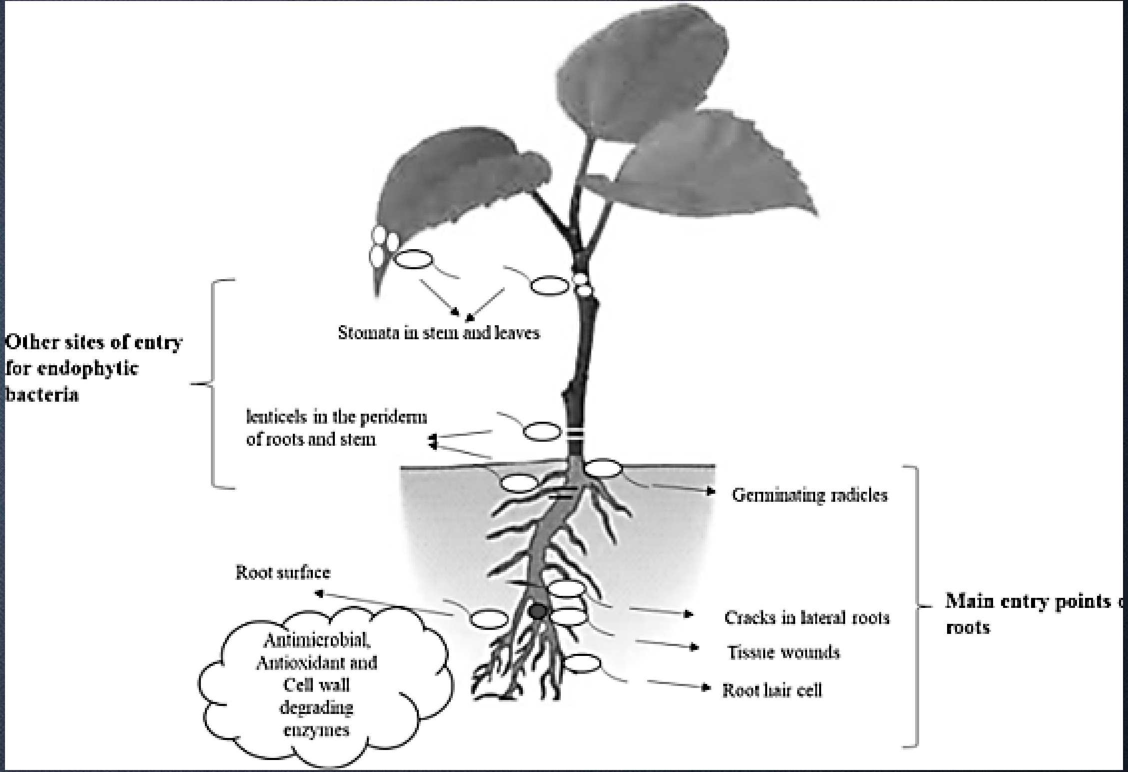


Previous studies

- ❑ Endophytic bacteria are usually found in the roots of plants, and studies have shown that these bacteria are also found on the leaf surface, stem, flowers, fruits and seeds.

- ➢ It has been determined that they can enter the plant through the gaps of the stem cells and root hairs, the scar tissues in the plant or the leaf stoma.

- ✓ Besides, it has been observed that they have the ability to move vertically in the plant.



- ❑ (Sessitsch et al. 2004, Berg et al. 2005, Okunishi et al. 2005, Rosenblueth and Martinez-Romero 2006).
- ➢ (Huang 1986, Agarwhal et al Shende 1987, Sprent and de Faria 1998, Sørensen and Sessitsch 2015).
- ✓ (Hallmann et al. 1997, James et al. 2002, Hardoim et al. 2008, Compant et al. 2010).

Previous studies

- In recent studies, it has been revealed that these bacteria are also found in seeds of some plant species, and in this regard, this can be an alternative route for colonization in the plant (Truyens et al. 2015, Lopez et al. 2018).

- Regarding this, according to Herrera et al. (2016) and Walitang et al. (2017), they determined that endophytic bacteria colonized in the ovaries and fruits of some plants and reported that it is possible to transfer the bacteria to the next generation with these seeds.

- Studies have shown that endophytic bacteria are found in the seeds of many agriculturally important plant species such as cotton, corn, wheat, rice and tomato.

- Information about the functions of endophytic bacteria in seeds is limited, and there are very few studies on the possible effects of these bacteria found in seeds on plant growth.

- (Misaghi and Donndelinger 1990, Hardoim et al. 2012, Gond et al. 2015, Herrera et al. 2016, Mitter et al. 2017, Lopez et al. 2018).

- (Compant et al. 2010, Truyens et al. 2015, Walitang et al. 2017, Lopez et al. 2018, Matsumoto et al. 2021).

Previous studies

- In this regard;

Verma et al. (2017) Rice
Mitter et al. (2017) Pepper, Wheat, Maize, Soy
Lopez et al. (2018) Tomato
Krishnamoorthy et al. (2020) In various aromatic plant species

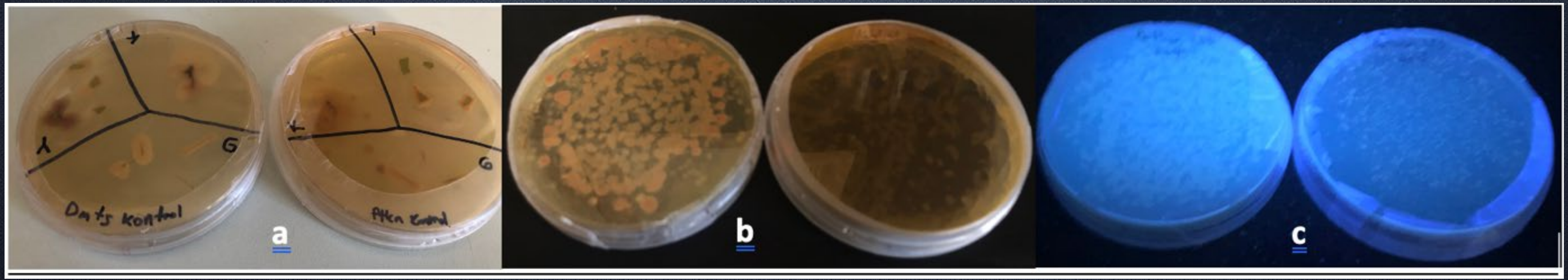
These researchers demonstrated effect of endophytic bacteria in seeds on the germination performance and their activities under various stress factors.

3 . Materials and Methods

Materials

❑ Endophytic bacterial species

✓ In the study, 4 different endophytic bacteria, two Pseudomonas (L5B and L13 strains) and two Bacillus (ApBm and Bs1) strains, which produce siderophores and synthesize indole acetic acid (IAA), were used.



REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

Materials

Species	Cultivar	Endophytic bacterial species
-Tomato	-Şencan 9	- *Pseudomonas floroscense* (L5B) - *Pseudomonas gessardii* (L13) - *Bacillus subtilis* (Bs1)
-Eggplant	-Pala 49	- *Bacillus mojavensis* (ApBm)

Şencan 9

Pala 49

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

Methods

- The study consists of the work packages listed below;

1- Production of seeds containing endophytic bacteria (Yalova)

2- Detection and quantification of the presence of endophytic bacteria transferred into the seeds (Ankara)

3- Examination of development of plants produced from seeds containing endophytic bacteria (Yalova)

TAGEM
R&D AND INNOVATION

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

1- Production of seeds containing endophytic bacteria

Soil preparation in the greenhouse → Soil analysis → Fertilization program → sowing seeds → Obtaining mature seedlings → Parcelization in the greenhouse → planting seedlings

↓

plant development → During the plant development period, in each plot; plant height, plant diameter, number of lateral branches Three times (June, July and August) measuring → Inoculation preparation → Inoculation to flowers

↓

Fruits harvested Days after anthesis;
For Tomato;
65±2 days

For Eggplant;
55±2 days

→ Extraction of seeds → Determination of seed moisture content → Detection of the presence of Endophytic bacteria in seeds

Determination of seed moisture content ↓ 7±0,5 %

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

1- Production of seeds containing endophytic bacteria

- The trial was repeated for two production years between 2020-2022.

TAGEM
R&D AND INNOVATION

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

1- Production of seeds containing endophytic bacteria

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

1- Production of seeds containing endophytic bacteria

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

1- Production of seeds containing endophytic bacteria

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

1- Production of seeds containing endophytic bacteria

2- Detection and quantification of the presence of bacteria transferred into the seeds

- In order to determine the presence of bacteria transferred to the seed, 1 g seed sample was taken. Seeds of each application taken at different harvest dates were first subjected to surface disinfection in 1% NaOCl for 10 minutes.
- Afterwards, the seeds were rinsed 3 times for 5 minutes in sterile distilled water and dried between sterile blotting papers. A suspension was obtained by homogenizing the seeds, whose surface disinfection was completed, in 5mL of 0.85% NaCl using Bioreba extraction bags in 0.85% NaCl.
- Up to 104 dilution series were prepared from the suspension of each seed lot, and 100 µl of NA, King's B medium and Triptic soy Agar (TSA) from these series were spread using a drigalski spatula.
- In order to determine whether there is any bacterial contamination from the seed surface, 100 µl of the last rinse water after surface disinfection of each seed lot was taken and planted in NA, King's B and TSA media with the help of a drigalski spatula.
- Seeds of the resulting suspensions were incubated at 28°C for 48 hours.
- In terms of being a morphological reference, the bacterial strains used in spraying were also used as a reference by sowing in the same medium.
- Colonies growing on the media were examined and purified.
- Obtained strains were also examined in terms of their fluorescence under UV light (366 nm).
- Single colony isolation was made from colonies that were similar to reference strains.
- In addition, all the different colonies formed were purified and stored in sterile NB containing 30% Glycerol in cryo tubes at -86°C.

2- Detection and quantification of the presence of bacteria transferred into the seeds

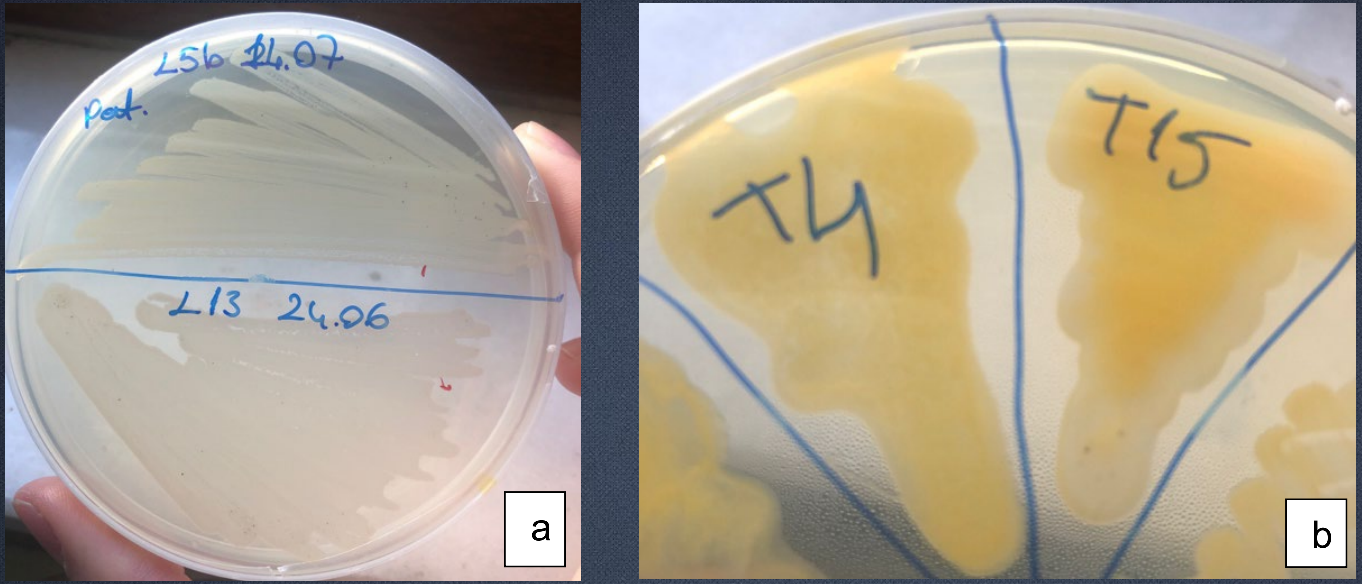


Figure 1. Pure colonies obtained from eggplant seeds after flower inoculation and showing flourescence in King B medium (a), colony structure formed by pure colonies obtained from tomato seeds and not showing flourescence in King B medium on nutrient agar (b).

2- Detection and quantification of the presence of bacteria transferred into the seeds

- DNA isolation was performed from cultures grown from single colonies obtained with a commercial DNA isolation kit.
- Primer pair 63f (5′-CAGGCCTAACACATGCAAGTC-3′) 1387r (5′-GGGCGGWGTG TACAAGGC-3′) was used to determine the 16S rRNA gene region in PCR of nucleic acids.
- According to the results of the sequence analysis, it was determined that Bacillus (ApBm and Bs1) strains of endophytic bacteria used in the project could not enter the seeds of both species through flower application, while Pseudomonas (L13 and L5b) strains successfully entered the seeds. Endophytic bacteria used in the experiment were not detected in control seeds.

- *Pseudomonas* (L13 ve L5b) ✔
- *Bacillus* (ApBm ve Bs1) ✘

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

2- Detection and quantification of the presence of bacteria transferred into the seeds

According to the sowing results from the dilution series, the fluorescent colony count was determined with the following equation and the arithmetic average was taken for 3 replications.

Number/ml = (Number of fluorescent colonies X Dilution Factor) / the volume spread from the dilution series to the petri dish (ml)

- Dilution Factor = 1 / Dilution rate

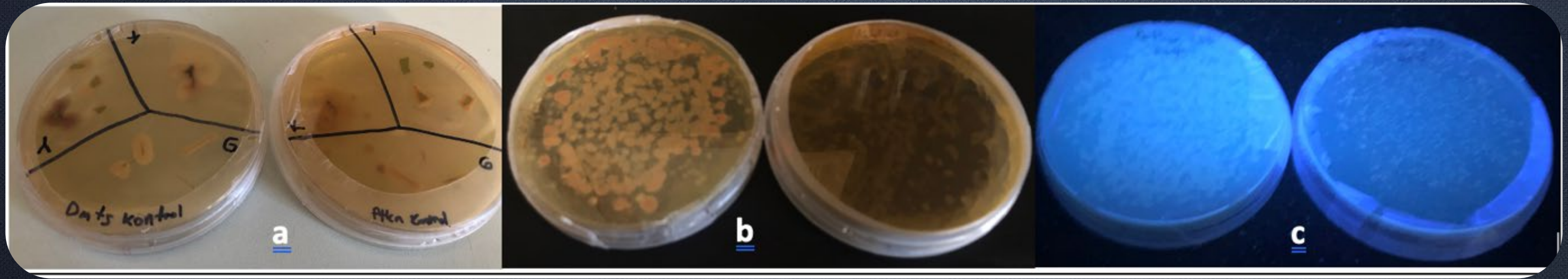


Figure 2 Petri dish evaluation for colony growth (a) and colony count on King B medium for different dilution series (visible light (a and b) UV light (c)).

3- Examination of development of plants produced from seeds containing endophytic bacteria

- The seedlings were grown at 23±1°C in a climate chamber with 16 hours of LED light, 8 hours of darkness and 60-70% humidity.

3- Examination of development of plants produced from seeds containing endophytic bacteria

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

3- Examination of development of plants produced from seeds containing endophytic bacteria

- Seed coating with bacteria: Control group seeds were coated with Pseudomonas (L13 and L5b) endophytic bacteria strains separately .

- According to Sankar et al. (2017); tomato and eggplant control group seeds were sterilized with 2% sodium hypochlorite for 30 seconds, then rinsed 3 times in distilled water and left to dry on sterile blotting papers overnight. 5 ml of the suspension of each bacterial strain, OD600: 108 CFU/ml inoculum was taken into a Petri dish, and then 100 mg of Carboxy Methyl Cellulose (CMC) was added as a binder. One gram of seed was added to this suspension and treated with the endophytic bacteria suspension for 2 hours in a shaker incubator, then placed in a sterile Petri dish and dried at room temperature overnight.

TAGEM R&D AND INNOVATION

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

3- Examination of development of plants produced from seeds containing endophytic bacteria

- The matured seedlings were planted in 8L pots with clay loamy soil in May. The pots were taken into the greenhouse and the development of the plants was followed for 60 days from planting. The experiment was established in a randomized plot design with 4 replications and each replication had 10 plants.
  - During the experiment period, the development of the plants was observed 3 times at regular intervals and evaluated in terms of "plant height", "stem diameter", "leaf number", and "cluster / flower number" criteria. In addition, the flowering dates of the plants were recorded and the transition times to the generative phase were compared.

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

3- Examination of development of plants produced from seeds containing endophytic bacteria

3- Examination of development of plants produced from seeds containing endophytic bacteria

- At the end of the experiment, the fresh and dry weights of the above – soil parts of the plants were measured.

Dry weight was determined by keeping the plants in the oven at 80°C for 24 hours.

4. Results and Discussion

TAGEM R&D AND INNOVATION

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

2- Detection and quantification of the presence of bacteria transferred into the seeds

Table 3. Amount of fluorescent bacteria detected in seeds containing endophytic bacteria (colony/CFU) (T: Repeat)

APPLICATION-Seed																	
L13						**L5B**						**CONTROL**					
TOMATO			EGGPLANT			TOMATO			EGGPLANT			TOMATO			EGGPLANT		
T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3
49	70	78	78	61	72	64	80	72	54	66	75	4	0	2	8	6	6

Table 4. Amount of fluorescent bacteria detected from seedlings obtained from seeds containing endophytic bacteria (colony/CFU) (T: Repeat)

APPLICATION-Seedling																	
L13						**L5B**						**CONTROL**					
TOMATO			EGGPLANT			TOMATO			EGGPLANT			TOMATO			EGGPLANT		
T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3	T-1	T-2	T-3
22	17	79	15	24	20	23	21	16	14	17	18	0	0	1	2	3	2

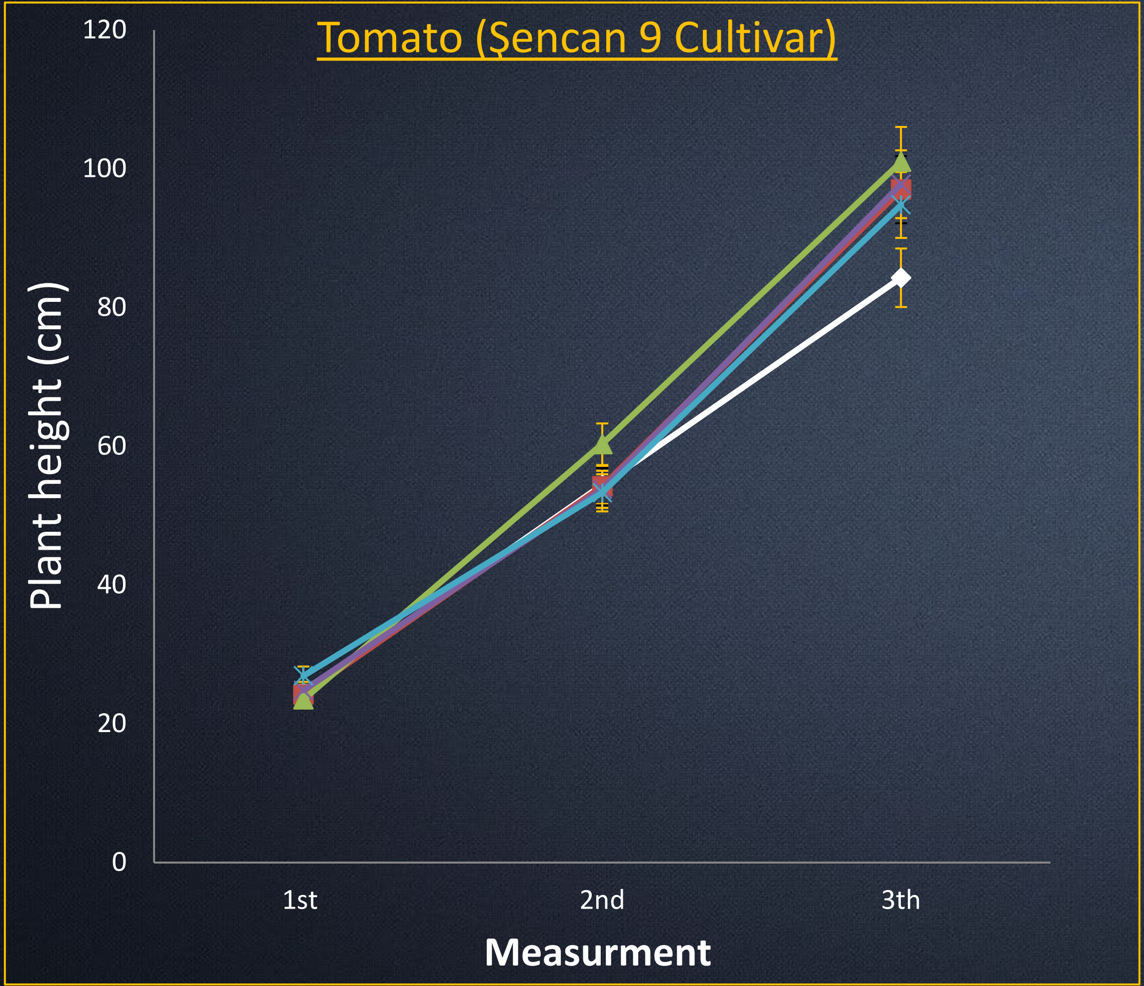
Table 5. The result of 16S rRNA gene region analysis of endophytic bacterial strains used in the study and bacterial strains obtained from flower applications (D: Tomato, P: Eggplant, DK: Tomato control group, PK: Eggplant control group represents seeds)

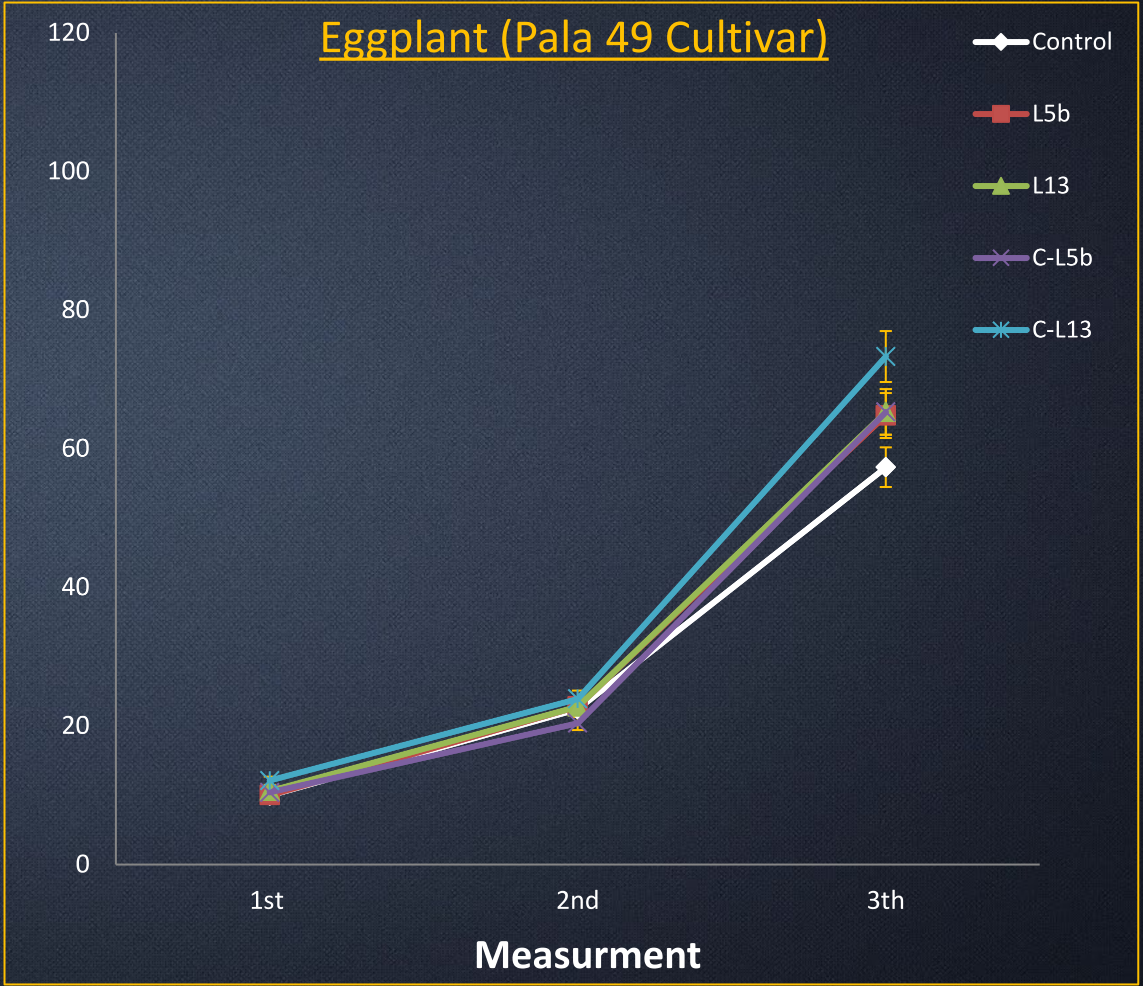
Strain no	Date of inoculation into flowers / inoculated strains	Sequence analysis result
ApBm	Reference strain used in the study	Bacillus mojavensis
Bs 1	Reference strain used in the study	Bacillus subtilis
L 13	Reference strain used in the study	Pseudomonas gessardii
L 5b	Reference strain used in the study	Pseudomonas fluorescence
D 7	24.06.2020 / L13	Pseudomonas gessardii
D 8	24.06.2020 / L13	Pseudomonas gessardii
D 9	24.06.2020 / L13	Pseudomonas gessardii
D 11	01.07.2020 / L5b	Pseudomonas fluorescence
D 12	01.07.2020 / L13	Pseudomonas gessardii
D 13	01.07.2020 / L13	Pseudomonas gessardii
D 14	01.07.2020 / L13	Pseudomonas gessardii
D 18	14.07.2020 / L13	Pseudomonas gessardii
P 4	24.06.2020 / L5b	Pseudomonas fluorescence
P 5	24.06.2020 / L5b	Pseudomonas fluorescence
P 6	24.06.2020 / L13	Pseudomonas gessardii
P 7	24.06.2020 / L13	Pseudomonas gessardii
P 14	01.07.2020 / L5b	Pseudomonas fluorescence
P 15	01.07.2020 / L 13	Pseudomonas gessardii
P 16	01.07.2020 / L 13	Pseudomonas gessardii
P 20	14.07.2020 / L5b	Pseudomonas fluorescence
P 21	14.07.2020 / L 13	Pseudomonas gessardii
P 22	14.07.2020 / L 13	Pseudomonas gessardii
DK 1	16.06.2020	Acetinobacter spp.
DK 2	24.06.2020	Acetinobacter spp.
DK 3	01.07.2020	Acetinobacter spp.
DK 4	14.07.2020	Enterobacter chloae
PK 1	24.06.2020	Pseudomonas paralactis
PK 2	01.07.2020	Pantoea agglomerans
PK 3	14.07.2020	Enterobacter spp.

TAGEM R&D AND INNOVATION

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

3- Examination of development of plants produced from seeds containing endophytic bacteria

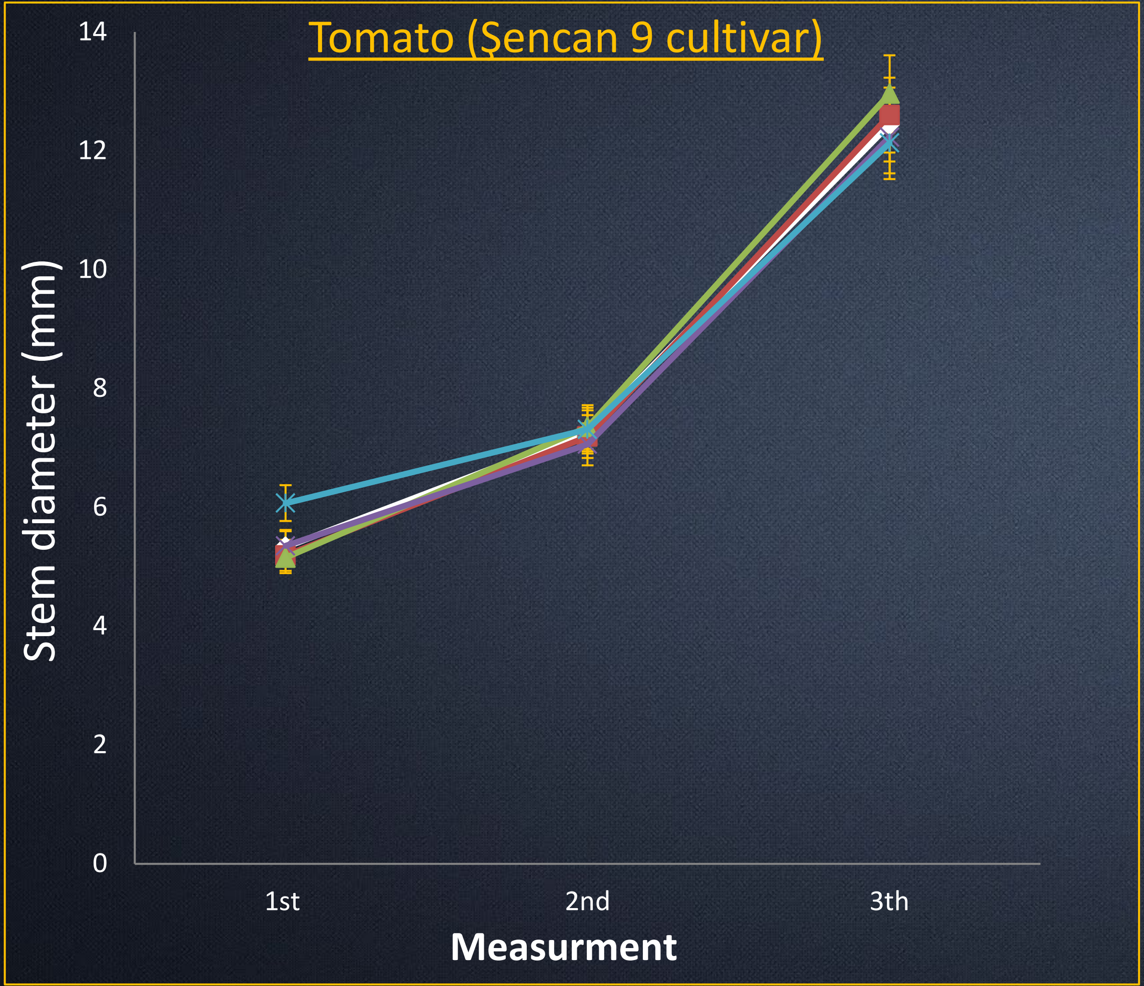


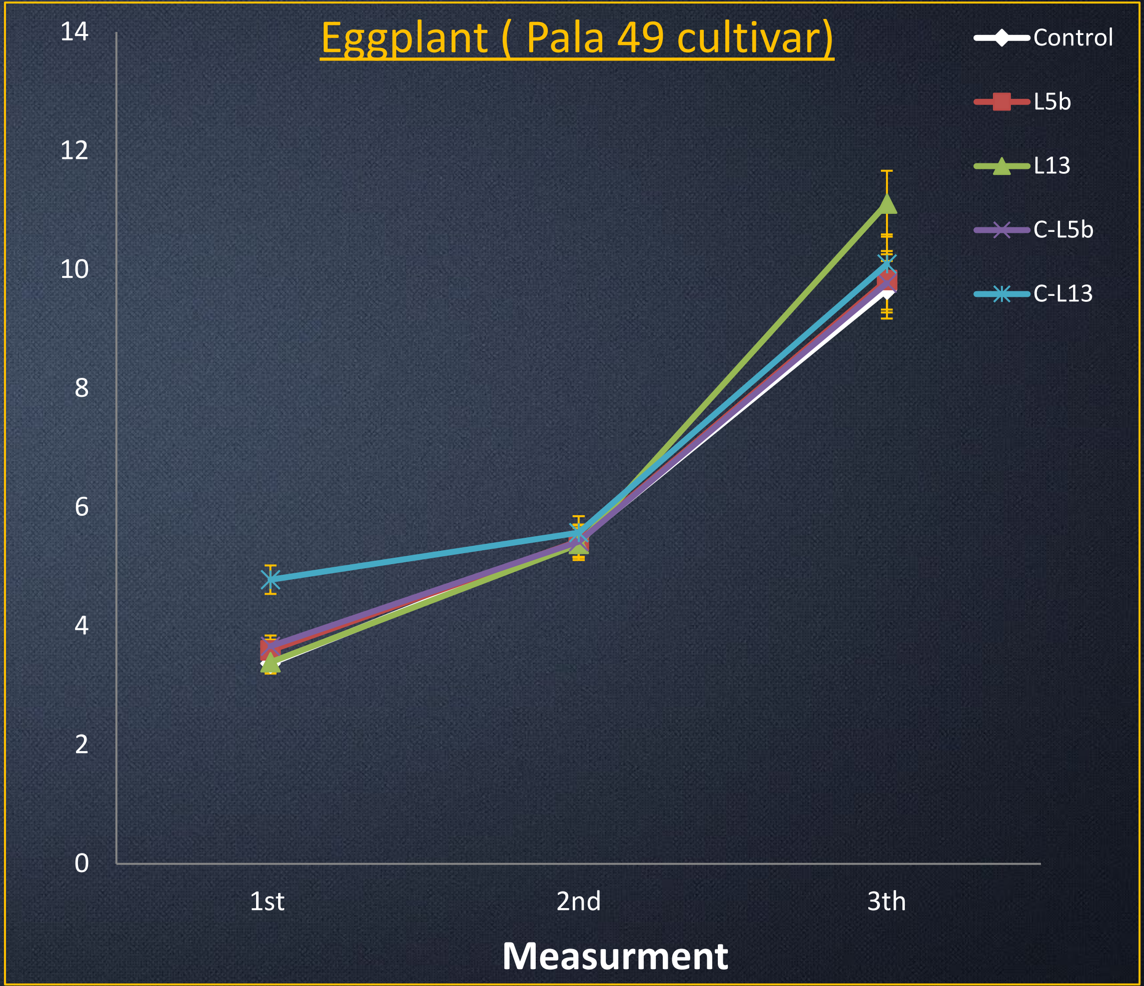


TAGEM
R&D AND INNOVATION

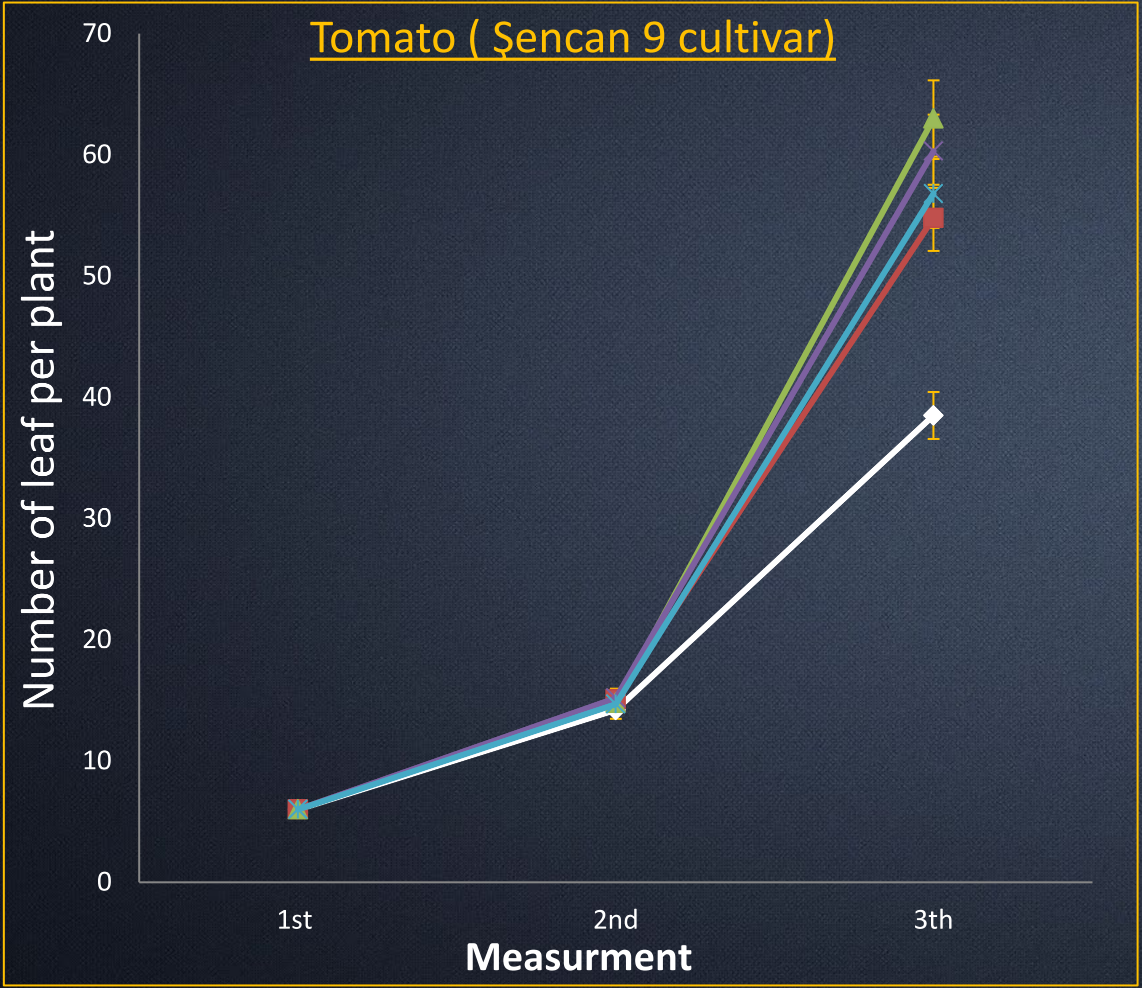
REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

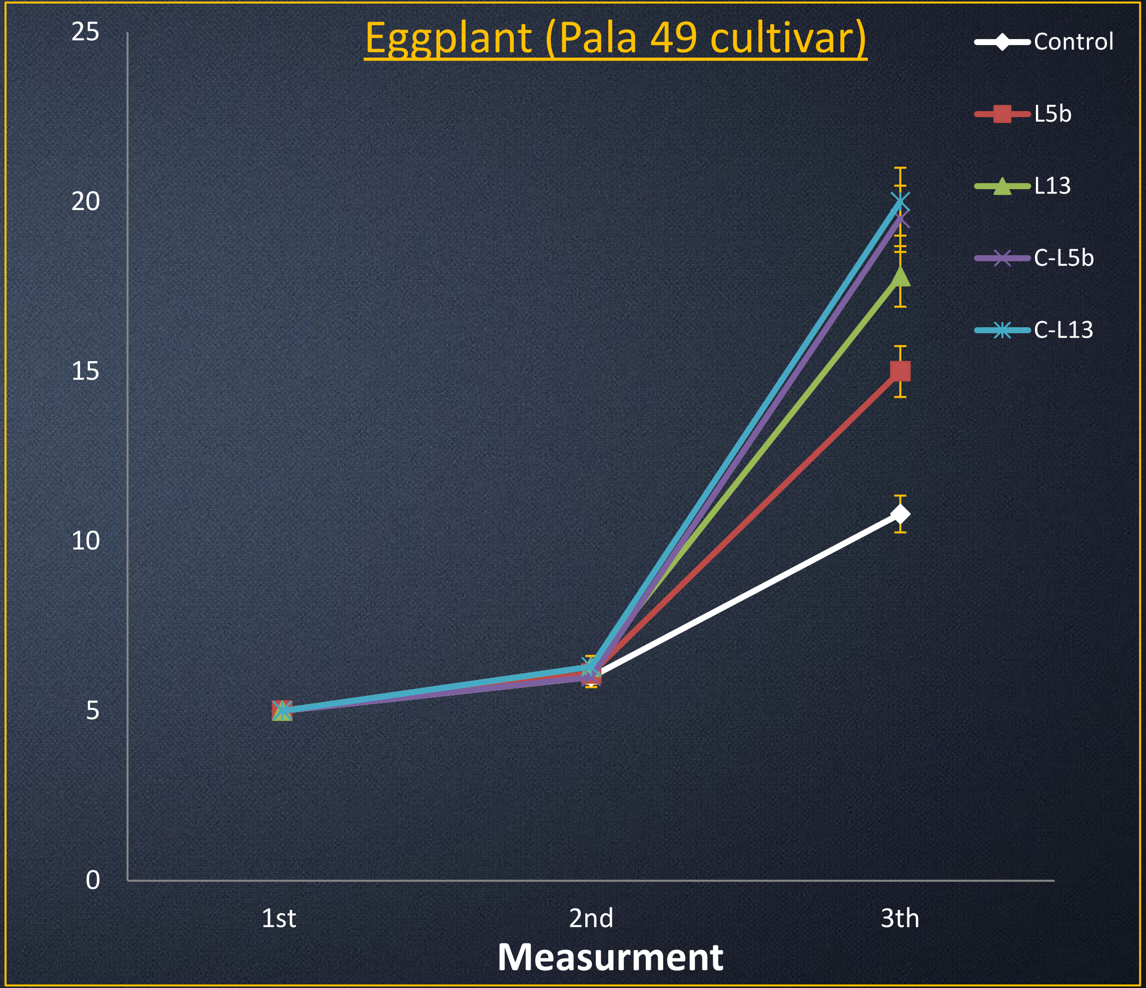
3- Examination of development of plants produced from seeds containing endophytic bacteria





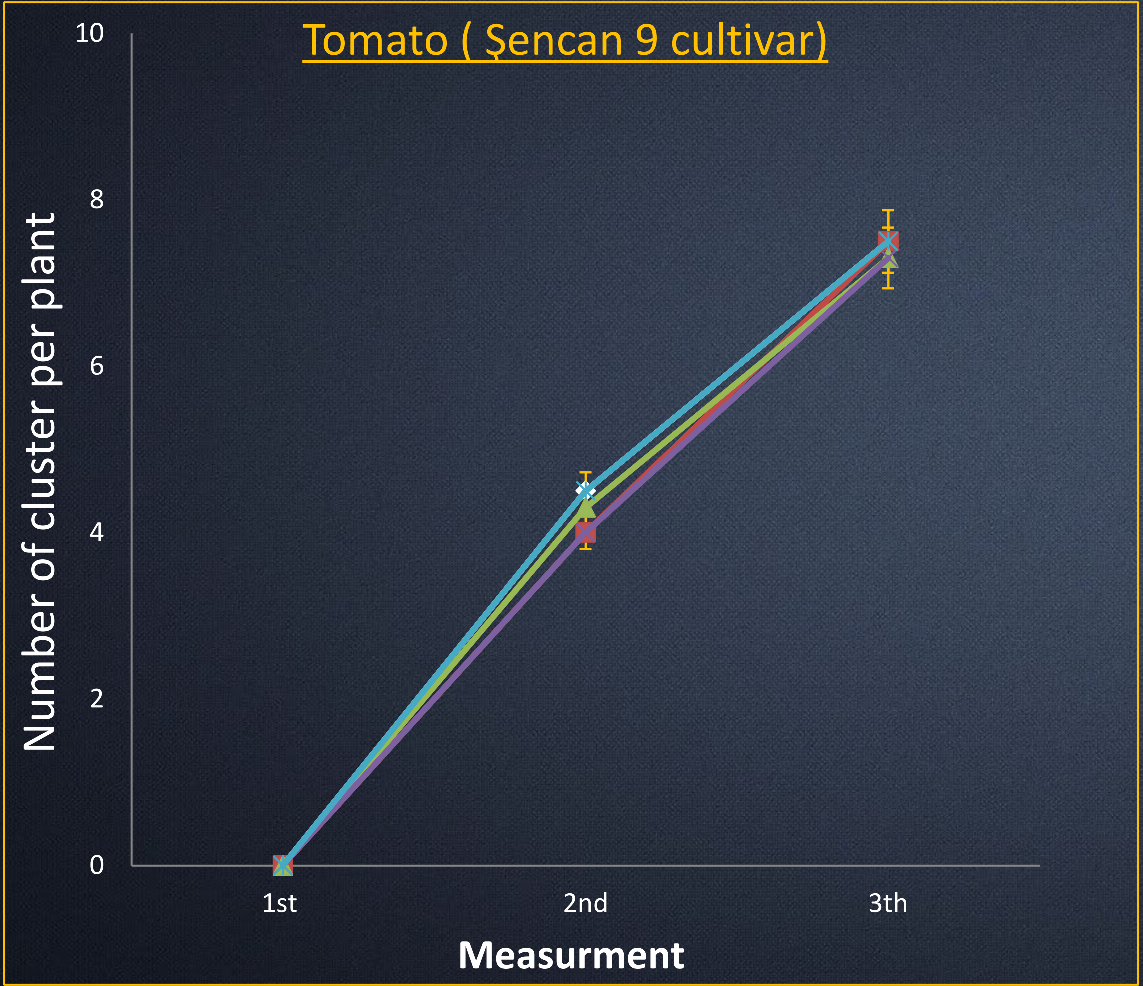
3- Examination of development of plants produced from seeds containing endophytic bacteria

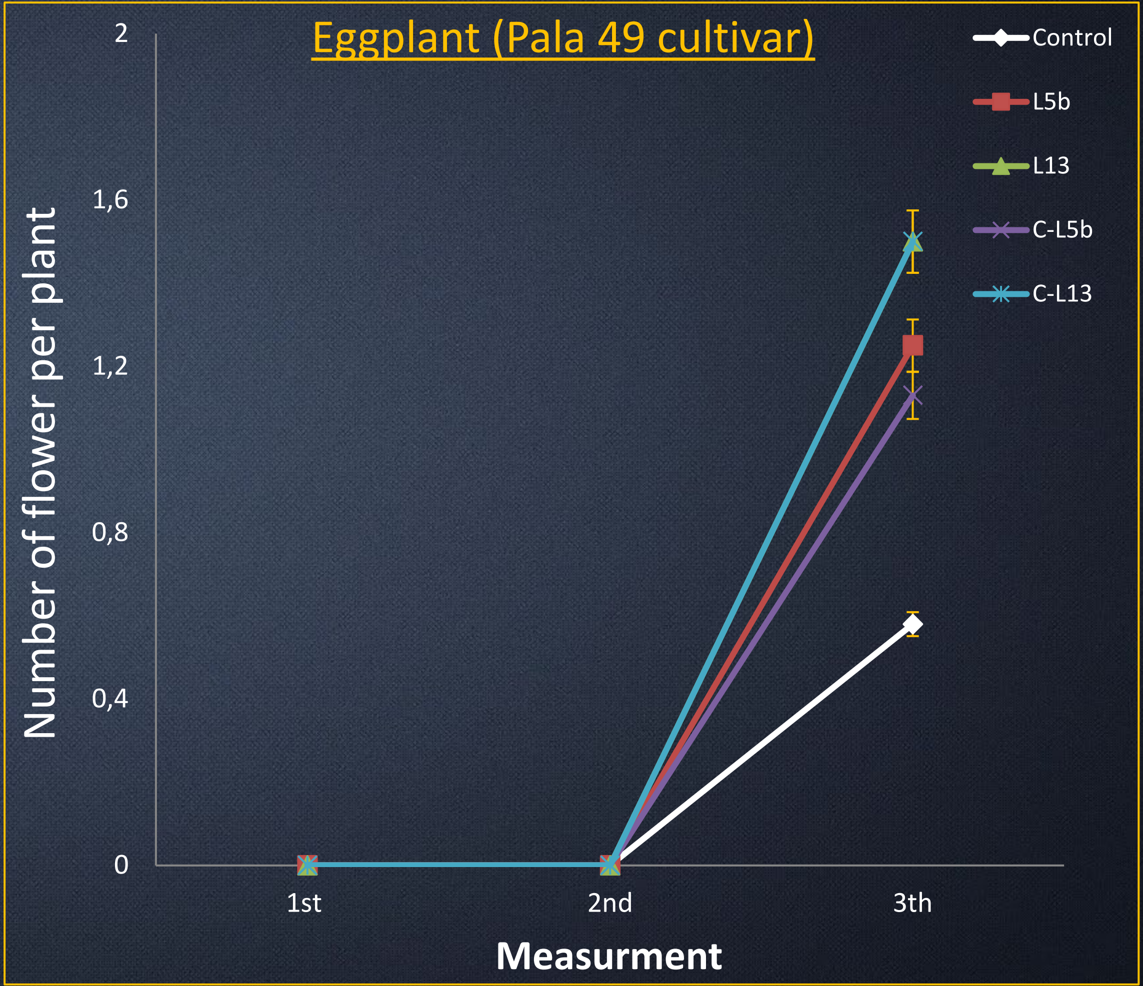




REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

3- Examination of development of plants produced from seeds containing endophytic bacteria





3- Examination of development of plants produced from seeds containing endophytic bacteria

Table 6. Time to reach the generative phase of seeds containing endophytic bacteria and seeds coated with bacteria (day)

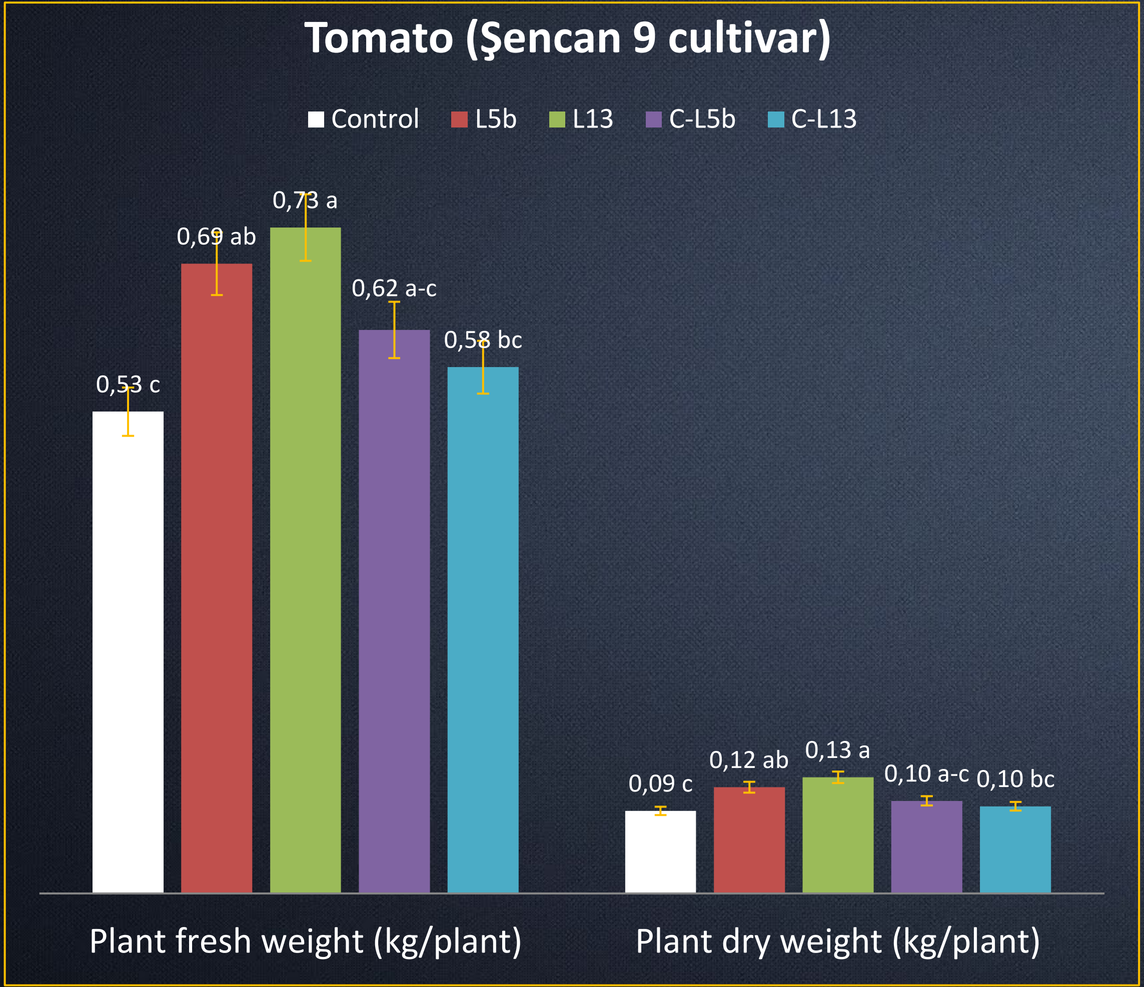
Seed lots	Time to reach flowering (From sowing to first flower seen (day))	
	Tomato (Şencan 9)	**Eggplant (Pala 49)**
Control	70,3	91,5
L5b	71,0	92,0
L13	70,8	91,5
C-L5b	70,5	91,3
C-L13	70,3	90,8
CV:	1,11%	1,31%
P:	N.S.	N.S.
LSD:	1,18	1,41

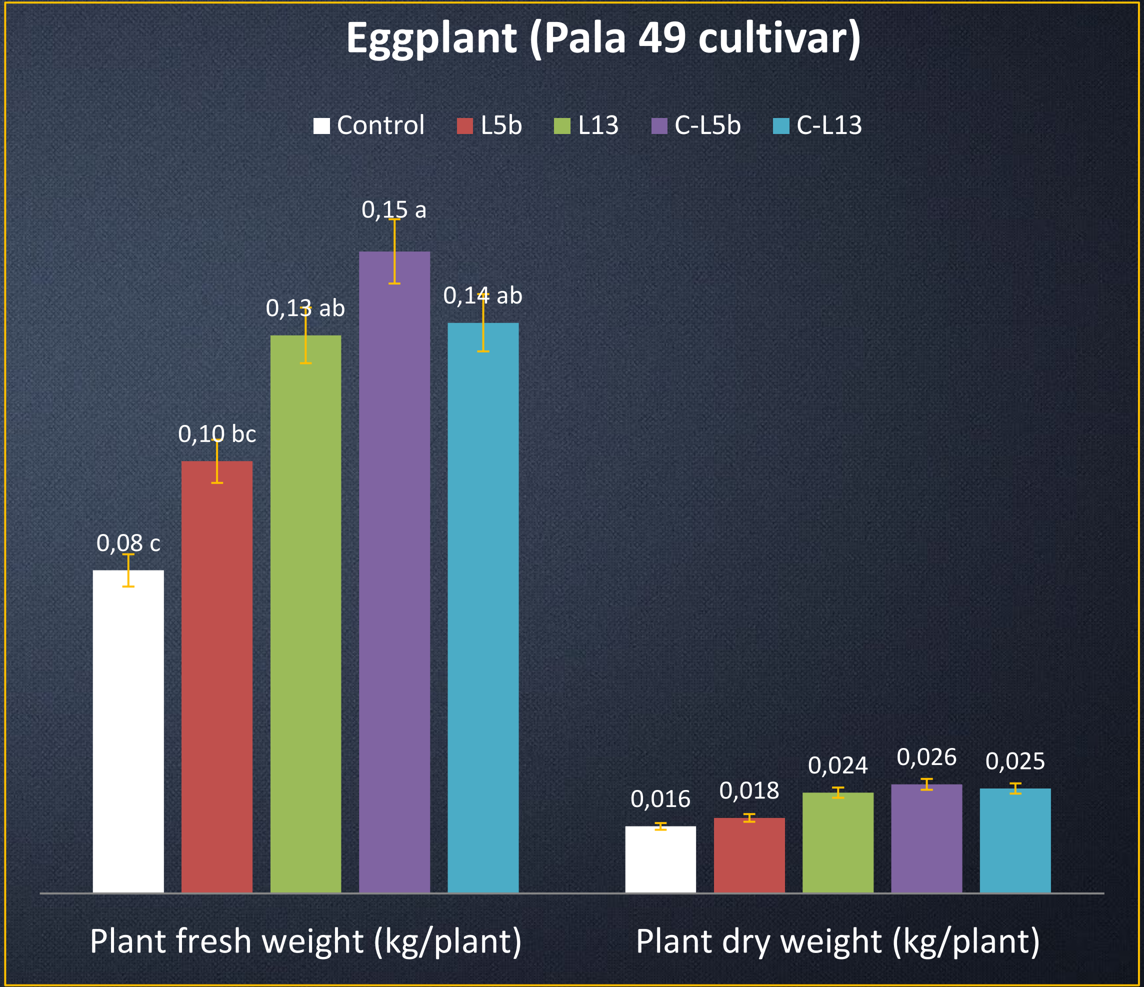
C-: Seed lot coated with endophytic bacteria, N.S: Not significant

TAGEM R&D AND INNOVATION

REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

3- Examination of development of plants produced from seeds containing endophytic bacteria





REPUBLIC OF TÜRKİYE
MINISTRY OF AGRICULTURE AND FORESTRY

Discussion and Conclusion

- ✓ Seed microbiota can have a crucial role for crop installation by modulating germination, seedling development, and recruitment of plant symbionts.
- ✓ Little knowledge is available on the fraction of the plant microbiota that is acquired through seeds.
- ✓ Studies have shown endophytic bacteria found in the seed promote germination, seedling development and plant growth.
- ✓ Regarding this, Verma et al. (2017) found that endophytic bacteria naturally found in rice seeds play an active role in seedling development.

- ➢ Compant et al. 2010, Truyens et al. 2015, Walitang et al. 2017, Lopez et al. 2018, Chesneau et al. 2020

Discussion and Conclusion

- ✓ The transfer of endophytic bacteria into seeds is a novel method and only one article has been found on the development of plants grown from seeds containing endophytic bacteria.

- ❖ In this study, by applying *Paraburkholderia phytofirmans* bacteria to wheat, corn, soybean and pepper flowers, the transfer of bacteria was successful from the stigma of the flower.

- ✓ In our study, Pseudomonas (L13 and L5b) strains were successfully transferred into tomato and eggplant seeds by inoculating into stigma. But Bacillus strains could not enter the seeds.

❖ Mitter et al. 2017

Discussion and Conclusion

- When the studies on the activities of endophytic bacteria on plant growth are examined;

- Composition of seed microbial community can modify colonization of wheat roots by dark septate endophytes, three weeks following germination (Ridout et al. 2019). Thanks to this effect, managing seed microbiota composition is a promising tool to improve plant growth.

- In a study conducted with wheat, higher yield, seed weight and plant growth were obtained in the experiment carried out under high temperature with the application of endophytic bacteria compared to the control (Park et al. 2017).

- In our study, the growth of plants produced with flower inoculated seeds were compared with the bacteria-coated seed lots in terms of plant development and biomass differences.

Conclusion

- ✓ There was an increase in biomass with endophytic bacteria treatments (flower inoculation and seed coating) in both species (tomato and eggplant) compared to the control group.
- ✓ Increase was determined in plant fresh and dry weights.
- ✓ In the development of plants; plant height, and number of leaves increased (especially in L13 strain).
- ✓ In point of the number of flowers, L13 strain came to the fore in eggplant.
- ✓ There was no difference found in terms of time to reach the generative phase of plants.
- ✓ When the relationship between flower inoculation and seed coating applications were examined; It can be said that there was no difference between the applications.

- This study was carried out within the scope of the project numbered 119O687 "The Production of Seeds Containing Endophytic Bacteria in Some Vegetable Species and the Investigation of the Resistance of These Seeds to Different Biotic and Abiotic Stress Factors"

❖ We thank "THE SCIENTIFIC AND TECHNOLOGICAL RESEARCH COUNCIL OF TÜRKİYE" for financially supporting this project.

Thank you for listening.

kutaycoskun.yildirim@tarimorman.gov.tr

Dr. Kutay Coşkun YILDIRIM