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AUTHORS: Ahmet AKKÖPRÜ, Kamuran ÇAKAR, Ahmad HUSSEINI

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Araştırma Makalesi/Research Article (Original Paper) Effects of Endophytic Bacteria on Disease and Growth in Plants under Biotic Stress

Ahmet AKKÖPRÜ*, Kamuran ÇAKAR, Ahmad HUSSEINI

Van Yüzüncü Yıl Univ., Agriculture Faculty, Plant Protection Department, Van, Turkey *Corresponding author: ahmetakkopru@yyu.edu.tr

Abstract: The aim of this study was to reveal the effects of four endophytic bacteria (EB) (Ochrobactrum sp. CB36/1, Pantoea agglomerans CC37/2, Bacillus thuringiensis CA41/1 and Pseudomonas fluorescens CC44) on the plant development of tomato and pepper and the effects against bacterial spot disease caused by Xanthomonas euvesicatoria (Xe) in both hosts. EB applied on tomato and pepper seedlings cultivated in a sterile peat growing medium in a climate chamber in two different periods to the roots. The pathogen inoculated on the leaves by spraving. Disease severity was measured by different scales for tomatoes and peppers, and plant development parameters were determined at the end of the study. The antagonistic effects of EB against Xe and 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase activities were determined with in vitro studies. The effect of endophytic bacteria on tomato and pepper varied according to the host plant x endophyte x pathogen combination. While no bacteria were effective against the pathogen in vitro, Ochrobactrum sp. CB36/1 inhibited the disease severity by 37% in tomato plants, but this effect was not observed in pepper. Tomato and especially pepper plants under disease stress had root and shoot fresh and dry weight increased by 28% to 128% by EB. The measurable effects of EB under biotic stress were determined to be higher than in stress-free conditions. In conclusion, the endophytic bacteria used in the study have potential for use within sustainable integrated agricultural concept framework, with their effects determined to vary according to the host, pathogen and endophytic bacteria.

Keywords: Bacterial spot disease, Pepper, PGPR, Tomato, Xanthomonas euvesicatoria

Biyotik Stres Altındaki Bitkilerde Endofit Bakterilerin Hastalık ve Bitki Gelişimi Üzerine Etkileri

Özet: Bu çalışmada dört endofit bakterinin (EB) (*Ochrobactrum sp.* CB36/1, *Pantoea agglomerans* CC37/2, *Bacillus thuringiensis* CA41/1 ve Pseudomonas fluorescens CC44), domates ve biberin bitki gelişimi ile her iki konukçuda *Xanthomonas euvesicatoria (Xe)*'nın oluşturduğu bakteriyel leke hastalığına karşı olan etkilerinin ortaya konması amaçlanmıştır. Steril torf ortamında iklim odasında yetiştirilen domates ve biber fidelerine EB'ler iki farklı dönemde köklere uygulanmıştır. Patojen yapraklara pülverize edilerek inokule edilmiştir. Domateste ve biberde farklı sıkalalarla ölçülen hastalık şiddeti ve bitki gelişim parametreleri deneme sonunda belirlenmiştir. Ayrıca *in-vitro* çalışmalar ile EB'lerin *Xe*'ye karşı antagonistik etkileri ve 1-Aminocyclopropane-1-carboxylic asit (ACC) deaminase faliyetleri belirlenmiştir. Endofit bakterilerin domates ve biberdeki etkisi konukçu Bitki x Endofit x Patojen kombinasyonuna göre farklılık göstermiştir. Hiçbir bakteri *in-vitro* da patojene karşı etkinlik göstermez iken *Ochrobactrum sp.* CB36/1 domateste hastalık şiddetini %37 oranında engellemiş, fakat biberde bu etki gözlenmemiştir. Domates ve özellikle biberde hastalık baskısı altında EB'ler kök ve sürgün yaş ve kuru ağırlıklarını %28 ile %128 oran aralığında arttırmıştır. Sonuç olarak çalışmada kullanılan endofit bakterilerin sürdürülebilir entegre tarım konsepti çerçevesinde kullanım potansiyelinin olduğu, bu etkinin konukçu, patojen ve endofit bakterilere göre değişebileceği belirlenmiştir.

Anahtar kelimeler: Bakteriyel leke hastalığı, Biber, PGPR, Domates, Xanthomonas euvesicatoria

Introduction

In tomato (*Solanum lycopersicum*) and pepper (*Capsicum annum*) production, bacterial spot disease caused by Xanthomonas species leads to significant product and quality losses. Bacterial spot disease can be formed by four different *Xanthomonas* species; *X. euvesicatoria, X. perforans, X. vesicatoria,* and *X. gardneri* (EPPO 2013).

Of these, *X. euvesicatoria* and *X. gardneri* infect tomato and pepper, while *X. perforans* infects tomato and *X. vesicatoria* strains generally are seen in tomato (EPPO 2013). Generally the disease causes spots on the leaves, stem and fruit, in addition to causing cracking of the stem and defoliation (EPPO 2013). Recent studies in Turkey have determined *X. euvesicatoria* is a commonly encountered species in some region (Eryigit 2016).

The controlling of disease with resistant varieties and cultural practice has not always provided the desired results, while the negative effects of pesticides on the environment and long known resistance problems have increased the importance of biological control. Within this framework, plant growth-promoting rhizobacteria (PGPR), and a recent focus within this bacteria group of endophytic bacteria (EB), have significant potential. EB are defined as bacteria which spend at least part of their lives living in the internal tissues of the plant and do not have negative effects on the plant (Rosenblueth and Martínez-Romero 2006; Hardoim et al. 2008).

PGPR or EB may affect the growth and development of the plant directly or indirectly. PGPR may produce the plant development hormones of indole-3-acetic acid, cytokinine, auxin (van Loon, L.C. 2007). In addition to these, they reduce the ethylene level which is harmful to plants, make nutritional elements into useable form and stimulate the resistance mechanisms of the plant directly contributing to plant growth and health (Saharan and Nehra 2011). Examples of the indirect effect mechanisms of PGPR may be given as plant protection as they also act as a biocontrol agent reducing the efficacy and quantity of pathogens, encouraging beneficial symbiotic relationships or decomposing xenobiotics found in soil (Saharan and Nehra 2011).

In addition to the mechanisms mentioned above, different to other PGPRs, EBs live in the internal tissues of plants ensuring a closer relationship with the plant. Additionally, colonization is not limited to a certain region of the plant and with transport to other tissues via the xylem and phloem transport system, this ensures the possibility of intervention against pathogens in all areas and with many mechanisms (Rosenblueth and Martínez-Romero, 2006; Hardoim et al. 2008). The interior tissues of the plant, are protected them from biotic and abiotic stress factors found in the external environment and this aids in sustaining their long-term presence. (Rosenblueth and Martínez-Romero 2006). Due to these advantages, many researchers have tested the efficacy of different pathosystems for *Fusarium oxisporum f.s cucumerum, Pseudomonas syringae* pv *lachrymans* (Özaktan et al. 2015) and *Pythium ultimum* (Benhamou et al. 2000) in cucumber, *Clavibacter michiganensis subsp. sepedonicum* (ring rot) (van Buren et al. 1993) in potato and *Setosphaeria turcica* (D'Alessandro et al. 2014) in corn and obtained different levels of success.

The aim of this study was to research the effects of four endophytic bacteria on the plant growth of tomato and pepper and to determine the effects against bacterial spot disease caused by the leaf pathogen *Xanthomonas euvesicatoria* in both hosts.

Materials and Methods

Plants, EB and Pathogen

Pepper (*Capsicum annuum* cv. Demre) and tomato (*Solanum lycopersicum* cv. Marmande) were used as plant material in the study. The four endophytic bacteria isolates showed PGPR activity in cucumber in previous study (Özaktan et al. 2015), were used as biological control agents (Table 1). The virulent pathogen on tomato and pepper was obtained from Dr. Hatice Özaktan.

Table 1. Endophyte bacteria isolates, IAA and siderophore production and phosphate (P) solubilizing ability determined in previous studies (Özaktan et al. 2015).

Endophyte bacteria	IAA (ppm)	Siderophore (mm)	P. solubilizing (mm)
Ochrobactrum sp. CB36/1	135	7	4
Pantoea agglomerans CC37/2	45	7	6
Bacillus thuringiensis CA41/1	6	1	0
Pseudomonas fluorescens CC44	8	6	1.5

Cultivation of plants, application of Xe and EB

Pesticides free pepper and tomato seeds were planted in 250ml volume containers filled with sterile peat and left in a climate room at 24±2 °C, 60% humidity 14 hour light conditions. During the study, nutrition required by seedlings was meet as recommended by Akköprü and Özaktan (2018).

With the aim of applying EBs to seedlings, 48-hour EB cultures developed on King's B medium (Pepton 20g/L, K₂HPO₄ 1,5g/L, MgSO₄7H₂O 1,5g/L, Glycerol 10ml/L, Agar 15g/L.) were prepared in suspension with 10^{8} cfu/ml density. The suspensions were applied twice to the seedlings by drenching method with 10ml/plant, the first EB application was performed when the first true leaves began to open with, the second application after the second true leaves opened (nearly four week seedlings).

The pathogen were applied once 4 days after the last EB application. With this aim, 48-hour *Xe* culture grown on KB medium was prepared in suspension with 10^8 cfu/ml and 0.5% Tween 80 was added as surfactant, and this was applied to the leaves with a hand sprayer. Immediately after pathogen application, the plants were left in polyethylene cabins with the aim of creating high relative humidity for 48 hours.

In-vitro studies

Effect of EB on Xe development: The 10^7 cfu/ml density suspension obtained from 48-hour *Xe* cultures (100 ul) was spread on the surface of KB medium. After the medium surface had dried, EBs were obtained from 24-hour cultures with the aid of a pointed loop and inoculated at four points on the KB medium. The results were obtained by measuring the zone with development of *Xe* prevented around the EB colonies.

Determination of ACC deaminase activity: With the aim of determining the ability of EBs to produce 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase that was reduced the ethylene levels formed during infection, a DF minimum salt medium (4.0 g/l KH₂PO₄; 6.0 g/l Na₂HPO₄; 0.2 g/l MgSO₄.7H₂0; 1.0 mg/l FeSO₄ 7H₂0; 10 ug/l H₃BO₃; 10 ug/l MnSO₄; 70 ug/l ZnSO₄; 50 ug/l CuSO₄; 10 ug/l MoO₃; 20 g/l Agar) (Dworkin and Foster 1958) was used. This medium also had 670 mg/L malic acid, 2 g/l glucose and 2 g/l citric acid as carbon source (Ribaudo et al 2016) and 2 g/l (NH₄)₂SO₄ as nitrogen source added (Penrose and Glick 2003). To determine the ACC deaminase activity, instead of the nitrogen source of (NH₄)₂SO₄, ACC (Merck KGaA) was used. ACC of 600 mg/l (6mM) dissolved in sterile pure water was sterilized with filtered, and it (100ul) was spread on the DF medium surface. After ACC was fully dried, the isolates to be tested were plated. The petri dishes were incubated at 28 °C for 48-72 hours and colony development observed. A DF medium was used as negative control.

Experiments in planta

Determination of disease severity; Three weeks after Xe application disease symptoms on tomato plants were assessed by scale 1-7 (1=no disease symptoms, 2=some necrotic spots on some leaflets, 3=some necrotic spots on many leaflets, 4=combined spots on some leaflets, 5=combined spots on many leaflets, 6=severe disease symptoms and defoliation, 7=plant death) (Abbasi et al 2002). For pepper plants, the disease severity ratings (0-6 scale) were based on the infected leaf area as follows: 1:no disease symptoms, 2: a few necrotic spots or <10% disease symptoms, 3: combined spots and common spots or 10-25%, 4: 26-50%, 5: 51-75%, 6: > 76% or fallen or dead leaves.

The disease index and efficacy (%) were calculated using the following formulas;

$$Disease index = \frac{\Sigma (Rating number x Number of leaves in the rating)}{Total number of leaves x Highest rating} x100$$

Efficacy (%) = $\frac{Control value - Treatment value}{Control value}$

Determination of plant development parameters; The effect of EB on tomato and pepper were determined in the 8th week when the study ended. Height measurement was obtained by measuring the length from the root collar to the growing tip. Total leaf counts were identified by counting each simple leaf on pepper and each compound leaf on tomato. After seedlings cut at the root collar had the root portions washed, roots and shoots were separately weighed to determine fresh weight. Then roots and shoots were dried in a drying oven at 65 °C for 72 hours and weighed again to obtain dry weights.

Analysis of Data; Experiment including treatments (1: NC, 2: PC, 3: tomato/pepper + EB, 4: tomato/pepper + EB+Xe) were set up according to completely randomised with ten replicates. The data were analysed using SPSS v17.0 statistical software. Significant differences between treatments were determined using Duncan's multiple range test with a significance level of $P \le 0.05$.

Results

In-vitro studies

At the end of *in vitro* studies, none of the EB isolates were determined to suppress or have direct antagonistic effect on *Xe* colonies. Additionally, ACC deaminase activity was not observed any EB isolates.

Disease severity

Data related to determination of the effect of endophytic bacteria on disease severity caused by *Xe* in tomato and pepper plants are shown in Fig. 1. The disease formation in tomato was limited by the CB36/1 isolate when compared to positive controls, and this effect was at the rate of 37% compared to the positive control. However, CA41/1, CC44 and CC37/2 isolates reduced disease compared to positive control; but, this effect was not statistically important. In peppers, it was identified that endophytic bacterial isolates did not have effects on disease development (Fig. 1).

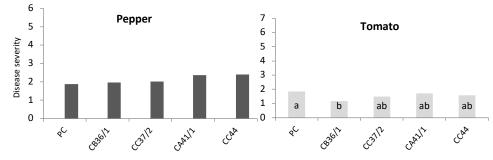
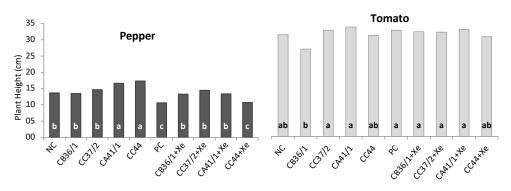
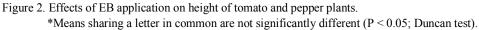


Figure 1. Disease severity in tomato and pepper plants with EB applied four weeks after Xe application. * Means sharing a letter in common are not significantly different (P < 0.05; Duncan test).

Effects of EB isolates on plant growing parameters

In pepper, alone treatments of CA41/1 and CC44 isolates increased plant height, while CB36/1+Xe, CC37/2+Xe, and CA41/1+Xe isolates increased plant height even under disease stress (Figure 2). In tomato, CC37/1 and CA41/1 isolates caused an increase, though not a significant difference; however, this effect was not observed with other applications. Applications in both plants were not determined to affect leaf numbers.





The effects of applications on plant shoot growth were obtained by weighing. In pepper plants without pathogen application, CC44 isolate significantly increased the shoot fresh weight, while other isolates did not display such high success though they were different compared to negative control (NC). Contrary to this, under disease stress, apart from CC44+*Xe* application, CB36/1 and CC37/2 applications significantly increased shoots fresh weight by the rate of 29%. For tomato plants, CC37/2 application reduced shoot fresh weight, while other EB applications had no effect on plants whether pathogen was applied or not (Fig. 3).

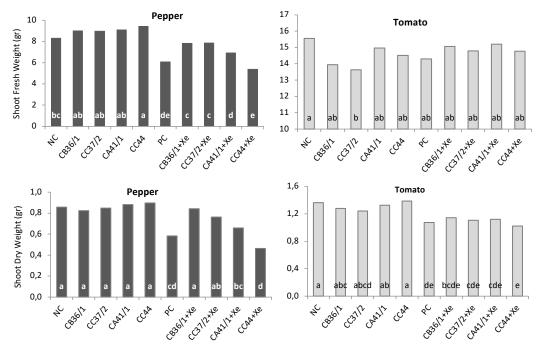


Figure 3. Effect of applications on shoot fresh weight of tomato and pepper plants. * Means sharing a letter in common are not significantly different (P < 0.05; Duncan test)

While EB isolates showed no effect on shoot dry weight without disease stress in pepper, under disease stress CB36/1+Xe (45%) and CC37/2+Xe (31%) isolates significantly increased dry weight. CA41/1+Xe application caused an increase but it was not at significant levels (Fig. 3). The significantly positive effect was not observed in tomato plants with and without disease stress.

When the EB isolates are applied alone, there was no positive contribution to the root fresh weight of pepper plants (Fig.4). Contrary to this, CB36/1+Xe application significantly increased the root fresh weight by the rate of 115 %, while CC37/2+Xe and CA41/1+Xe applications created noteworthy positive differences. In tomato plants without disease stress, the root fresh weights had no significant difference, while a positive increase was observed with disease stress (Fig. 4).

Similar results were obtained for pepper and tomato in terms of root dry weight. Without disease stress in pepper, EBs did not contribute to the root dry weight compared to NC, while under disease stress CB36/1+Xe application provided a significant degree of increase at the rate of 129% compared to PC. CC37/2+Xe and CA41/1+Xe applications were observed to provide a positive contribution. In tomato plants, application of EBs alone did not provide significant contribution, while EB+Xe applications provided a positive contribution compared to Xe alone treatment (Fig. 4).

When the effects of EB application on the plant growth parameters of plant height and fresh and dry weights of root and shoot are generally assessed, under disease stress CB36/1, CC37/2 and CA41/1 isolates were determined to provide more positive contribution to the plants (Tab. 2). Though this efficacy was observed in tomato plants, it was greater in pepper plants and at statistically significant levels. Apart from plant height, CC44 isolate had no efficacy. However, in pepper plants CB36/1, CC37/2 and CA41/1 isolates had high effect, with CC37/2 and CA41/1 isolates more successful in tomato plants though at low levels.

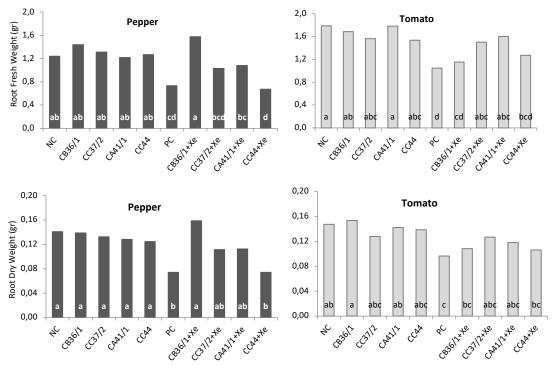


Figure 4. Effect of EB application on tomato and pepper root fresh and dry weights. *Means sharing a letter in common are not significantly different (P < 0.05; Duncan test).

Treatments / Parameters		CB	<u>CB36/1</u>		<u>CC37/2</u>		<u>CA41/1</u>		<u>CC44</u>	
i urunie	015	Xe-	Xe+	Xe-	Xe+	Xe-	Xe+	Xe-	Xe+	
РН	Pepper Tomato	-14	24*	4	35*	21* 7	25*	26*	- 6	
SFW	Pepper	9	29*	8	29*	10	14	-14*	- 11	
	Tomato	-10		-12*		4		7		
SDW	Pepper		45*		31*		14		- 21	
	Tomato	6	7	9	4	2	5		- 5	
RFW	Pepper		115*		41		48		- 8	
	Tomato	-6	10	-13	43*		52*	-15	21	
RDW	Pepper		129*		57		57			
	Tomato	5	11	-13	31		22	-5	9	

Table 2. Effects (%) on plant growth parameters of EB applications under the disease stress

Xe-: No pathogen applied, Xe+: Pathogen applied, PH: Plant Height, SFW: Shoot fresh weight,

SDW: Shoot dry weight, RFW: Root fresh weight, RDW : Root dry weight.

*: Means sharing a letter in common are not significantly different (P < 0.05; Duncan test).

Discussion

In this study, four endophytic bacteria were used against bacterial spot disease caused by *X. euvesicatoria* the leaf pathogen in tomato and pepper plants. Additionally, the efficacy of the endophytic bacteria on plant growth parameters under disease stress and against the same pathogen (*Xe*) was investigated in tomato and pepper plants.

While PGPRs colonizing the rhizosphere can suppress a pathogen causing disease localized in the phyllosphere by affecting the plant nutritional balance, plant resistance and tolerance, in addition to these, endophytic PGPRs can also suppress the pathogen using other biological control mechanisms due to their systemic distribution. The using Endophytic bacteria in this study (*Ochrobactrum sp.* CB36/1, *P. agglomerans* CC37/2, *B. thuringiensis* CA41/1 and *P. fluorescens* CC44) have been used against to *F.o f.sp. cucumerinum* and *Ps.* pv lachrymans

infection in cucumbers and successful results have been obtained (Özaktan et al. 2015). Additionally, many researchers have investigated the efficacy of different endophytic bacteria on a variety of pathosystems and obtained neutral or different levels of positive effect (Kang et al 2007; Muthukumar et al. 2010).

Romero et al (2016) determined the efficacy of different endophytes on tomato growth and some diseases at neutral or different levels. Similarly, Streptomyces, Bacillus and Pseudomonas species were determined to have different levels of effect on bacterial spot disease and plant growth in tomato (Naue et al. 2014) and pepper plants (Mirik et al. 2008). Our results comply with previous studies in showing the variation in effect on disease and plant growth depending on EB and host. EB application reduced the severity of bacterial spot disease in tomato plants, with no such effect observed in pepper plants (Figure 1). Romero et al (2016) determined that some endophytic bacteria had antimicrobial activity against Ps pv. tomato in in vitro and the disease has been suppressed by some of them in tomato plant via this route. On the other hand, in our study, the most successful isolate in suppressing disease was Ochrobactrum sp. CB36/1 (37% effect) but it was not determined to limit Xe development in vitro. This leads to the consideration that the effect on the pathogen and disease was not through direct biological control mechanisms, such as competition and antiobiosis, but may be ensured by contributing to plant nutritional balance and increasing plant tolerance or activation of plant resistance. Researchers determined that plant resistance in peppers had been triggered by endophytic Bacillus pumilus INR7 (Yi et al. 2013), Pseudomonas rhodesiae and Pantoea ananatis (Kang et al. 2007), and the disease caused by X.a. pv. vesicatoria was suppressed by rates of 52%, 34.7% and 26.3%, respectively. Ribaudo et al (2016) identified that EB application reduced symptoms in tomato as a result of activating ethylene hormone genes and SI-ACS genes related to pathogenicity in the plant. In addition to Ochrobactrum lupine KUDC1013 limit disease caused by X.a py. vesicatoria in pepper plants by stimulating plant resistance (Hahm et al. 2012).

Another mechanism that may affect disease appearance is that PGPRs increase plant tolerance reducing the formation or effects of disease symptoms (van Loon 2007; Akköprü and Özaktan 2018). The key fact of plant tolerance is the contribution to plant growth or health. Within this framework, data and observations related to plant development parameters obtained in this study strengthen this perception. Growth parameters varied according to EB isolate and plant, and the effect was observed to be positive or neutral. Though EBs did not have a significant effect on plant height in tomato plants, contrarily in pepper plants some isolates caused a significant level of increase (Fig. 2). Similarly, though many studies have taken the increase in plant height as a marker of PGPR effect (Kang et al. 2007; Muthukumar et al. 2010; Xia et al. 2015), Huang et al (2017) proposed that this parameter alone was not sufficient to assess the PGPR effects. In our study, the root and shoot fresh and dry weights in plants with EB applied were observed to increase by 28 to 128% (Tab. 2). Similarly, Xia et al (2015) stated that different endophytic bacteria increased growth parameters by mean 25% in tomato. This significant increase obtained in current study may be considered the result of changes in the plant nutrition and hormonal balances. The first thing that comes to mind in these situations is that the ACC deaminase enzyme produced by PGPRs may prevent harm by disintegrating the ACC that is the precursor of ethylene (Penrose and Glick 2003; Glick 2014). As it is well known that the ethylene increase linked to stress harmed or limited development of the plant (Glick et al. 2014). However, the EB isolates used in our study were not determined to have ACC deaminase activity. Ribaudo et al (2016) stated that the effects of endophytic bacteria on tomato and pepper growth may be due to the IAA they produce. Khan et al (2012) showed that EBs which produce IAA and have nitrogen fixation ability increased the growth, flowering and yield of many plants, including pepper and tomato. The endophytic Pseudomonas spp. (Muthukumar et al. 2010), Bacillus spp. and Serratia spp. (Amaresan et al. 2012) were determined that they increased the vigour index of root and shoot growth via secondary metabolites like IAA, siderophore and inorganic phosphate solvent enzymes, etc. When it is analyzed that the CB36/1 and CC37/2 isolates with successful results obtained produce IAA and siderophore and have phosphate solvent ability (Table. 1), it is considered that the increase observed in plant development parameters and tolerance may be due to these metabolites.

However, interestingly especially when under disease stress, EB tretments were observed to have greater positive effects on plant growth (Tab. 2). CB36/1 and CC37/2 application to peppers under disease stress had a greater positive effect compared to disease-free peppers, while similar results were observed for CC37/2 and CA41/1 isolates in tomato plants though at lower levels (Tab. 2). The CC44 application to both plants showed no positive efficacy under disease stress. Hardomim et al (2008) stated that the contribution of endophytes may be clearer under stress conditions. Barak et al (2006) in a study using endophytic bacteria against cold stress observed that the contribution of EB isolates was higher under stress factors. Endophytic bacteria may trigger significant physiological changes modulating plant growth and development (Conrath et al. 2006). The proportion of different endophytic bacterial groups were determined to significantly change following pathogen infection (Bulgari 2012). Additionally, apart from quantitative change, it is reported that some stress factors may affect the characteristics of endophytes (Tobita et al. 2013). In light of these studies stress factors may affect the

relationship between endophytes and plant and this may be reflected in the host plant in different forms. The observation of higher positive effect on the host under biotic stress in this study may be due to the Host x EB relationship or a variation in the activity of EBs. Additionally, the increase in tolerance stimulated in the plant via endophytic bacteria may have come to the forefront in plants under stress; thus, the positive effects may be reflected more in diseased plants compared to healthy plants.

In conclusion, the effect of four endophytic bacteria varied according to host plant. The Ochrobactrum sp. CB36/1 limited disease caused by *Xe* in tomato significantly, but any endophytic bacteria showed no effect in pepper. However, in pepper plants they were determined to significantly increase growing parameters in plants under disease stress. In this way, endophytic bacteria have the potential for use in a sustainable integrated agricultural concept framework; however, it must be considerd that this effect varies depending on the host, pathogen and endophytic bacteria.

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