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DETERMINATION OF ACC DEAMINASE, NITROGENASE AND ANTAGONISTIC ACTIVITIES OF GRAM NEGATIVE BACTERIA ISOLATED FROM WHEAT FIELDS AND THEIR SALT TOLERANCE

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ABSTRACT

1-aminocyclopropane-1-carboxylate (ACC) deaminase activity is an important marker for bacteria to support plant growth by lowering ethylene levels in plants. The enzyme has been found in limited numbers of bacteria and plays an important role in supporting plant growth and development under environmental stress conditions, by reducing stress induced ethylene production in plants. The aim of this study is to investigate strains with ACC deaminase, nitrogenase and antagonistic activities.

In the present study, bacteria isolated from rhizosphere soils and wheat seedlings from Canakkale were screened for ACC deaminase activity, and eight isolates were determined to be ACC deaminase positive. The results of traditional methods showed that eight bacteria were identified as *Pseudomonas*, *Enterobacter* and *Serratia* strains. ACC deaminase activity was determined by measuring the production of α-ketobutyrate. Nitrogenase activity was analyzed by Acetylene Reduction Assay (ARA).

According to the results, there are some differences in ACC deaminase activities among all strains isolated from wheat fields. ACC deaminase activity was analyzed on eight bacteria. The highest ACC deaminase activity was found on *Pseudomonas* sp. CKB55 (2833 nmol α-ketobutyrate.mg^{-1}.h^{-1}). The highest nitrogenase activity was determined on *Pseudomonas* sp. CKB10 (808 nmol ethylene/ mg.h). In antagonistic activity studies, *Pseudomonas* sp. CKB52 has shown an maximum inhibition zone against *Erwinia caratovora* ECC100, and the zone was 11 mm. Most of the isolates have shown tolerance to 1000 mM NaCl concentration.

It can be said that ACC deaminase- contaning bacteria could be an environment-friendly and promising potential strategy to promote plant growth, alleviate biotic and abiotic stresses and provide sustainable agriculture, especially for ethylene-sensitive plants production. As a conclusion, Gram negative bacteria, isolated from wheat fields, have high ACC deaminase, nitrogenase and antagonistic activities. Additionally, these bacteria have a high salt tolerance.

KEYWORDS:
Bacteria, ACC Deaminase Activity, Nitrogenase Activity, Antagonistic Activity, Salt Tolerance

INTRODUCTION

Today's world population is increasing, and new researches are still going on, in order to obtain higher yields from the unit area in order to ensure the feeding of this population. The purpose of agricultural production is to produce efficient, quality and reliable products. Many commercially produced chemicals are used to increase plant yield and to combat plant diseases. Microorganisms gain resistance against these chemicals and the chemicals cause environmental pollution, therefore negatively affect the plant, animal and human health [1]. Therefore, there is a need for agricultural practices, which do not disrupt the ecological balance of the environment [2].

In our country, which has a rich variety of agricultural products, due to its geographical features and climatic characteristics, the predominant agricultural crops are composed of cereals. Wheat, which is 40% in the field of field crops and is the most cultivated grain, has 225 million hectares of cultivation area in the world, 685 million tons of production and 3038 kg / ha of average yield [3]. There are biotic and non-biotic factors that cause significant losses in wheat yield. Non-biotic stress factors cause an avergage loss of yield of more than 50% of the plant, and the primary cause of loss of agricultural crops in the world [4].

One of the effects of these factors is the accumulation of ions, such as Na^+ and Cl^- in the soil, resulting in salt stress, which negatively affects the growth of the plant. Often salt stress is stimulating the production of ethylene, known to be a stress hormone [5,6]. In recent studies, it has been suggested that normal growth may occur under salt stress in plants, vaccinated with plant growth promoting rhizobacteria (PGPR) which contain ACC deaminase. Ethylene is an important growth hormone that is produced in most plants, expressed as mediators of different reactions and developmental

processes in plants [7, 8]. Ethylene can have both limiting and stimulating effects [7].

PGPRs in the rhizosphere region that stimulate the growth of the plant, because of the presence of ACC deaminase activity and the wide range of domains, such as the application of the chemical is more accurate [9, 10, 11, 12, 13]. These features make the selection of PGPR that exhibit ACC deaminase activity more reliable than other alternatives. ACC deaminase catalyzes the conversion of ACC to α-ketobutyrate and ammonia, by controlling the production of ethylene in the plant [14]. ACC deaminase has been identified in many microbial species, such as Gram-negative bacteria [15, 16], Gram-positive bacteria [17, 18], endophytic bacteria [19, 20], *Rhizobium* [21, 22, 23] and fungi [24, 25]. The researchers first studied ACC deaminase on *Pseudomonas* spp. and, in particular, these studies are based on the aim of reducing the negative effects of stress, by promoting plant growth under stress conditions [26, 27, 28, 29]. Several studies have shown that rhizobacteria exhibiting ACC deaminase activity have an antagonistic effect against microbial pathogens. In these studies, it is a widely used strategy to treat plant seeds and roots with bacteria that stimulate plant growth with ACC deaminase activity and can be used as biocontrols. For example, the damage caused by *Pythium ultimum* in cucumber and *Erwinia carotovora* in potatoes was prevented by the use of biocontrol agents with ACC deaminase activity [15]. Similarly, Yuquan et al. [30] reported that, ACC deaminase activity strains showed strong antagonistic effect against the *Fusarium oxysporum* pathogen [31, 32, 33, 34].

Nitrogen cannot be used directly by biological systems and therefore it is involved in soil and plant systems by biological and chemical fixation [35]. Some microorganisms convert the atmospheric nitrogen (N_2), which cannot be used by plants by biological nitrogen fixation, into the form of NH_4 which can be used with the help of nitrogenase enzyme [36, 37]. The conversion of free nitrogen in the air to ammonia by microorganisms is referred to as nitrogen fixation. Nitrogen, the enzyme that catalyses nitrogen fixation, was first obtained in 1966 by purification from the *Azotobacter vinelandii* by Bulen and Le-Comte [38]. *Clostridium*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Serratia*, *Agrobacterium*, *Xanthomonas*, *Bacillus*, *Enterobacter*, *Klebsiellla*, *Sinorhizobium*, *Acinetobacter*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Phosphobacteria*, *Glucanacetobacter*, *Burkholderia* species are among the bacteria with high biofertilizer potential [39, 40, 41, 42, 43, 44, 45, 46, 47, 48].

Recent studies show that microbiological factors are the sources that meet the nutrients, needed by the plant as biological fertilizers and plant stimulants. The production of these microorganisms and their metabolites in industrial terms and their use in agriculture is regarded as an environmentally friendly approach in terms of the sustainability of the ecosystem [2]. Therefore, the aim of this study was to determine ACC deaminase, nitrogenase and antagonistic activities of Gram negative bacteria, isolated from wheat fields and to investigate salt tolerances.

MATERIALS AND METHODS

Organisms. The bacteria with ACC deaminase activity were isolated from the samples of wheat seedlings and rhizosphere soil (about 15 cm depth) from Çanakkale / Biga Koruoba Village. The organisms used in the antagonistic activity studies were five microorganisms. These bacteria were *Pseudomonas tomato* Pt52-a, *Agrobacterium vitis*, *Rathayibacter tritici* DSMZ7486, *Rathayibacter iranicus* DSMZ7484, *Erwinia caratovora* subsp. *caratavora* ECC100. All species were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Germany) and Plant Protection Central Research Institute (Ankara). The bacteria used in antagonistic activity studies were cultured in Nutrient Broth (NB) (Merck) medium at 37 °C for 24 hours [49]. In this study, *Pseudomonas putida* DSMZ291, which is also used as a positive control, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) Culture Collection (Germany). This bacterium was cultured for 24 hours at 37 °C in King B medium.

Sterilization of Plant Surface. All root and leaf samples of the wheat plant were kept in 95% ethyl alcohol for two minutes and kept in 1% sodium hypochlorite (NaOCl) for one minute. It was then rinsed six times with sterile distilled water and prepared for the work [50].

Isolation and Identification of Organisms. After the roots of the wheat seedlings were shaken, 1 g of soil was taken from the soil and the appropriate dilutions were made. Then 100 µl from dilutions was taken to Nutrient Agar (NA) and the inoculations were made by spread plate method and the plates were incubated at 37 °C for 24 hours. Random selections were made from different colonies that developed at the end of the incubation period and after a series of passages, were inoculated into Nutrient agar (NA) medium and stored in refrigerator conditions. Furthermore, the wheat roots and leaves which were subjected to surface sterilization were crushed with the help of mortar and shaken into sterile water. The obtained solution was inoculated into the NA medium and used for isolation. All cultures were examined according to cell morphology and Gram staining characteristics and Gram-negative bacteria were used for the study.

Biochemical tests (Oxidase test, motility test, Glucose fermentation test, H_2S production, lactose fermentation, gelatinase activity, etc.) have been applied on these cultures and identification of these bacteria on the basis of morphological and biochemical assays was performed by Assoc. Prof. Dr. Gulten Okmen [51].

Qualitative Determination of ACC Deaminase Activities of Bacterial Cultures. Gram-negative bacteria, selected for use in the study, were inoculated into the Dwork-Foster medium (DF) and improved in this medium, containing ACC as a single nitrogen source [52, 53, 54]. Cultures were incubated for 24 hours at 37 °C in media containing ACC and, at the end of the incubation period, grown cultures were considered ACC positive.

Optimization of Cultures with ACC Deaminase Activity. Different temperature, pH, shaking speed and time studies were conducted to determine the environmental conditions essential for the best growth of eight bacteria, with ACC deaminase activity.

Effect of Temperature on the Growth of Cultures. All cultures were inoculated into NB media to determine the effect of temperature on bacterial growth. In this study, 25 ml serum bottles that contain 10 ml NB medium have been used. 100 µl of active cultures were inoculated into these flasks at different temperature values (28, 37 and 45° C), followed by incubation for 18 hours (Nüve EN500, Binder USA, Stuart Scientifica UK). At the end of the incubation period, growth was recorded as dry weight (mg/ml). All trials were conducted independently and three parallel.

Effect of pH on the Growth of Cultures. 100 µl active cultures were inoculated into serum flasks, containing 10 ml NB at different pH values (6, 7 and 8). Cultures were allowed to incubate at their optimum temperature for 18 hours. At the end of the incubation period, bacterial growth was determined as dry weight (mg/ml). The pH of the media was adjusted with the aid of pH meter (Thermo, USA) using 1N NaOH and 1N HCl. All trials were conducted independently and three of them parallel.

Effect of Agitation Speed on the Growth of Cultures. All of the isolates were inoculated into NB media at optimal temperature and pH values to determine the effects of shaking speed on the growth of cultures. 100 µl of active cultures were inoculated to the media prepared to be 10 ml of NB in 25 ml serum flasks at different agitation speeds (100, 200 and 300 rpm), and were incubated for 18 hours. At the end of the incubation period, bacterial growth was determined as dry weight (mg/ml). All trials were performed independently and simultaneously.

Effect of Incubation Period on the Growth of Cultures. All cultures were inoculated into NB media at optimal temperature, pH and agitation speed in order to determine the best growth time of bacteria. 100 µl of active cultures were inoculated to the NB medium, which had been prepared as 10 ml NB in 25 ml serum bottles at different time intervals (from one to five days). At the end of the incubation period, the time-dependent growth of the cultures was followed and dry weights (mg/ml) were recorded. All experiments were carried out independently and simultaneously.

Quantitative Determination of ACC Deaminase Activity. 100 µl of active cultures with ACC deaminase activity were inoculated into NB media (10 ml), and the cultures were centrifuged after 24 hours of incubation, under optimum conditions. The pellet was washed two times with physiological saline, then suspended into JNFB (7.5 ml) medium containing 5 mM 1-aminocyclopropane-1-carboxylate (ACC), and the next steps were performed according to [55]. The amount of alpha-ketobutyrate was recorded by measuring the absorbance values at 540 nm. Alpha-ketobutyrate concentrations were calculated from the standard curve. Enzyme activity was expressed in nmol alpha-ketobutyrate/dry weight x hours. *Pseudomonas putida* DSMZ291, known to have ACC deaminase activity, was included in the trial as a positive control.

Effect of Salt Stress on the Growth of Cultures. In this study, to determine the salt tolerance of cultures, all cultures were exposed to different NaCl concentrations (10, 25, 50, 100, 200, 300, 400, 800 and 1000 mM). 100 µl of active cultures were inoculated into the 10 ml of NB medium, with different NaCl concentrations. Cultures were incubated under optimal growth conditions. At the end of the incubation period, the growth of all cultures was determined as dry weight (mg/ml). The experiments were carried out in three parallel ways. In addition, the experiments were performed with positive control organism, *Pseudomonas putida* DSMZ291. At the end of this study, cultures with a high salt tolerance have been included in the next experiments.

Determination of Antagonistic Activity as *in Vitro.* The antagonistic activities of cultures were determined against yield spoilage pathogens, such as *Pseudomonas tomato* Pt52- a (30 °C), *Agrobacterium vitis* (30 °C), *Rathayibacter tritici* DSMZ7486 (30 °C), *Rathayibacter iranicus* DSMZ7484 (30 °C), *Erwinia caratovora* subsp. *carotavora* ECC100 (30 °C). The strains used in the

study were obtained from cultural collections of DSMZ and Central Research Institute of Plant Protection. Cultures used in antagonistic activity studies were activated in NB (Merck) medium at 37 °C for 18 hours and their density was adjusted to 0.5 McFarland. The inoculum ratio of the cultures was set to 100 µl. The active cultures (100 µl) with ACC deaminase activity were inoculated to plates containing Mueller Hinton Agar (MHA). The cultures have been inoculated toward the center from the edge of plate, then the plates were allowed to diffuse for 10 minutes. At the end of this period, the yield spoilage pathogen cultures were inoculated against bacteria with ACC deaminase activity with a 90-degree angle. All of the plates were incubated at 30 °C for 24 hours, and microbial interaction was examined. The antagonistic activity was recorded as an inhibition zone (mm) [56].

Determination of Dry Weight. All cultures developed at the medium for 37 °C for 24 hours were harvested in logarithmic phase. The harvested pellet was filtered through Whatman GF/C filters, with a diameter of 0.45 µm and dried for 24 hours at 50 °C [57].

Determination of Nitrogenase Activity. Nitrogenase activities have been measured to determine the biofertilizer potentials of cultures, known to have ACC deaminase activity. The amount of ethylene was calculated by applying acetylene reduction technique to all cultures [58]. Cultures were developed under optimal environmental conditions, afterwards the vials were closed with plastic plugs and covered with parafilm. 1 ml of acetylene gas was injected into the medium and the cultures were incubated again under the experimental conditions and afterwards 10 µl gas samples were injected to the gas chromatography and its data was read. The results were calculated as nmol ethylene/dry weight x hour (nmol ethylene/mg.h). The gas chromatography (Agilent J6W GC) used during the analyzes was set to following values: DB-1 column and flame ionized detector (FID), the column temperature to 100 °C, the temperature of the injection room to 100 °C and the detector temperature to 120 °C. The pure ethylene gas (99.9%) was used as the standard and the flow rate of the helium gas was adjusted to 1.4 ml / min.

RESULTS

Soil samples from wheat fields were used for the isolation of bacteria with ACC deaminase activity. Cultures grown in suitable media were examined, and colonies, which were developed in different morphological structure and color, were purified by passaging under aseptic conditions. The number of isolates obtained was 61 and 24 of them were Gram-negative and 37 were Gram-positive bacteria. The studies were carried out on Gram-negative bacteria. To determine qualitatively the ACC deaminase activity of the 24 Gram-negative bacteria isolated from wheat fields, a salt medium containing ACC (DF) was used as the only nitrogen source. The ACC deaminase activities of all cultures were determined by inoculation to this medium. The bacteria which showed growth at the end of the incubation period, was evaluated as ACC deaminase positive (Figure 1).

FIGURE 1
Qualitative ACC Deaminase Activities of Gram Negative Bacteria

Considering the results obtained in this study, qualitative ACC deaminase activities of 24 Gram-negative bacteria were investigated and only eight bacteria were found to be ACC positive (Table 1). Biochemical tests were applied to the cultures, in order to identify the eight Gram-negative and bacil bacteria which were determined to be positive for ACC deaminase activity. These tests were applied to the cultures, which include a motility test, IMVIC test, oxidase test, nitrate reduction test, glucose fermentation, fluorescent diffused blue pigment, H_2S production, lactose fermentation, gelatinase activity and urease activity. These cultures were identified after the tests (data not shown).

TABLE 1
Qualitative Results of ACC Deaminase Activities at Gram Negative Bacteria

Isolates	CKB2	CKB5	CKB8	CKB9	CKB10	CKB11	CKB16	CKB13
ACC deaminase activity	-	-	-	+	+	-	-	-
Isolates	CKB17	CKB18	CKB22	CKB25	CKB32	CKB33	CKB37	CKB44
ACC deaminase activity	+	-	-	-	-	+	-	-
Isolates	CKB45	CKB46	CKB49	CKB50	CKB52	CKB55	CKB56	CKB58
ACC deaminase activity	-	+	-	-	+	+	-	+

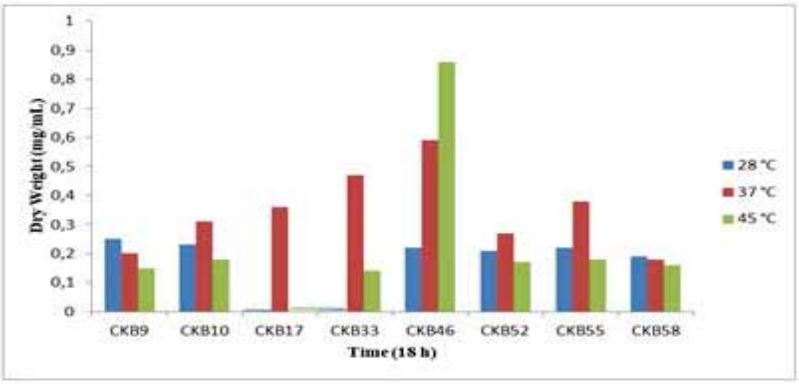

FIGURE 2
Effect of Temperature on the Growth of Cultures with ACC Deaminase Activity

The effects of different environmental factors were investigated, in order to determine the best growth conditions for the eight Gram-negative bacteria with ACC deaminase activity. These are different temperature, pH, agitation speed and time (Figures 2, 3, 4, and 5). The effects of different temperature values (28, 37 and 45 °C) on the growth of eight Gram-negative isolates were investigated. Considering the data obtained at the end of the study, only one isolate was optimally produced at 28 °C, and this bacterium is *Pseudomonas* sp. CKB9. Isolates showing optimum improvement at 37 °C were in total six, which are *Pseudomonas* sp. CKB10, *Enterobacter* sp. CKB17, *Serratia* sp. CKB33, *Pseudomonas* sp. CKB52, *Pseudomonas* sp. CKB55 and *Pseudomonas* sp. CKB58. At 45 °C, only *Pseudomonas aeruginosa* CKB46 could be developed optimally. According to this study, the optimum temperature for 75% of the isolates, was determined as 37 °C (Figure 2).

The effects of different pH values (pH 6, 7, 8) on the growth of isolates were investigated. Considering the data obtained from this study, the number of bacteria showing optimum growth in the application of pH 6 is one, and this bacterium is *Pseudomonas* sp. CKB52. In the application of pH 7, three bacteria showed optimum growth. These are *Serratia* sp. CKB33, *Pseudomonas aeruginosa* CKB46 and *Pseudomonas* sp. CKB58. When the values, obtained as a result of pH 8 application were taken into consideration, it was determined that four bacteria showed optimum growth. These are *Pseudomonas* sp. CKB9, *Pseudomonas* sp. CKB10, *Enterobacter* sp. CKB17 and *Pseudomonas* sp. CKB55. When different pH values were examined, the optimum pH for 50% of the isolates was determined as 8 (Figure 3).

Considering the studies on the effect of different agitation speeds on growth, eight Gram-negative bacteria, with ACC deaminase activity were found to be affected differently. According to this study, the growth of all strains was inhibited at 300 rpm. The highest growth of 200 rpm belongs to *Pseudomonas aeruginosa* CKB46 (2.16 mg/ml). The best growing cultures at 100 rpm were *Pseudomonas* sp. CKB9 (0.41 mg/ml) and *Serratia* sp. CKB33 (0.56 mg/ml). Six of the cultures showed a high growth of 200 rpm applications. Among these, the highest growth belongs to *Pseudomonas aeruginosa* CKB46 with 2.16 mg/ml. Other cultures that showed the best growth of 200 rpm, were *Pseudomonas* sp. CKB10, *Enterobacter* sp. CKB17, *Pseudomonas* sp. CKB52, *Pseudomonas* sp. CKB55 ve *Pseudomonas* sp. CKB58 (Figure 4).

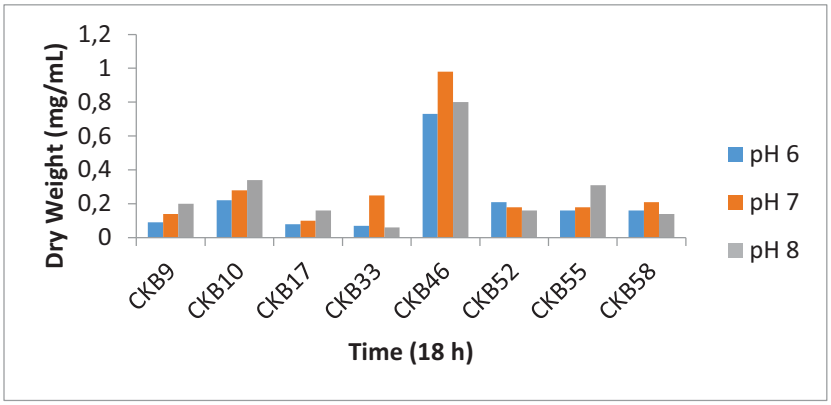

FIGURE 3
Effect of pH on the Growth of Cultures with ACC Deaminase Activity

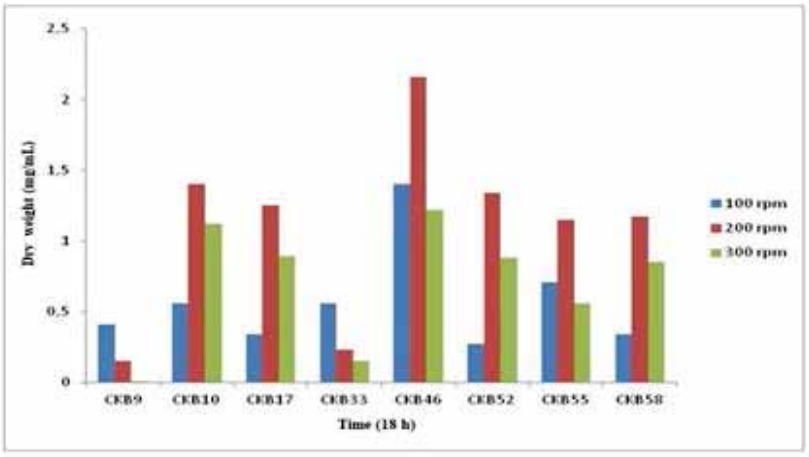

FIGURE 4
Effect of Agitation on the Growth of Cultures with ACC Deaminase Activity

In this study, eight Gram-negative bacteria, isolated from wheat fields and known to have ACC deaminase activity, were allowed to incubate under their optimal conditions (temperature, pH, rpm). The active cultures (100 µl) were inoculated to the prepared media under optimum conditions, and incubated at different time intervals (1, 2, 3, 4, 5 days). At the end of the incubation period, cultures were recorded as dry weight (mg/ml). Considering the data obtained from the study, 63% of the cultures showed optimum growth at the end of the first day, and 12% of the cultures showed the optimum growth at the end of the 4th day. At the end of the first day, the best growth showing cultures were *Pseudomonas* sp. CKB10, *Enterobacter* sp. CKB17, *Pseudomonas aeruginosa* CKB46, *Pseudomonas* sp. CKB52 ve *Pseudomonas* sp. CKB58. Subsequent studies were continued at the optimum growth times and conditions of their cultures (Figure 5).

The active cultures with ACC deaminase activity were inoculated into the NB medium, and these cultures were incubated under their optimal conditions for 24 hours. Then cultures were harvested, and pellets were put into the JNFB medium, that contain 1-aminocyclopropane-1-carboxylate (ACC), and the amount of alpha-ketobutyrate was calculated. The amount of ACC deaminase activity was recorded as nmol α-ketobutyrate.mg^{-1}.h^{-1}. In this study, *Pseudomonas putida* DSMZ291 known to have ACC deaminase activity, was used as a positive control. Considering the data obtained from this study, the highest ACC deaminase activity was determined from *Pseudomonas* sp. CKB55 (2833 nmol α-ketobutyrate.mg^{-1}.h^{-1}). The activity obtained from this culture is higher than *P. putida* DSMZ291 (1975 nmol α-ketobutyrate.mg^{-1}.h^{-1}), which is a positive control. Only 25% of the cultures had high ACC deaminase activity, while 75% of the cultures had less than the control value (Figure 6).

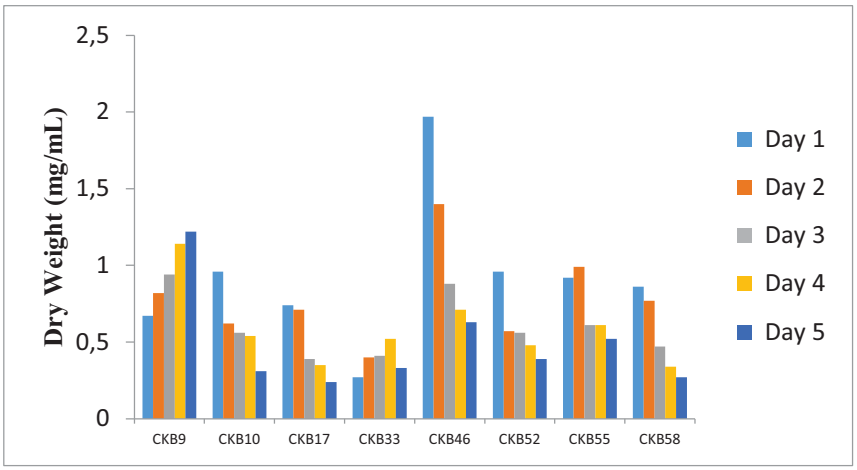

FIGURE 5
Effect of Incubation Period on the Growth of Cultures with ACC Deaminase Activity

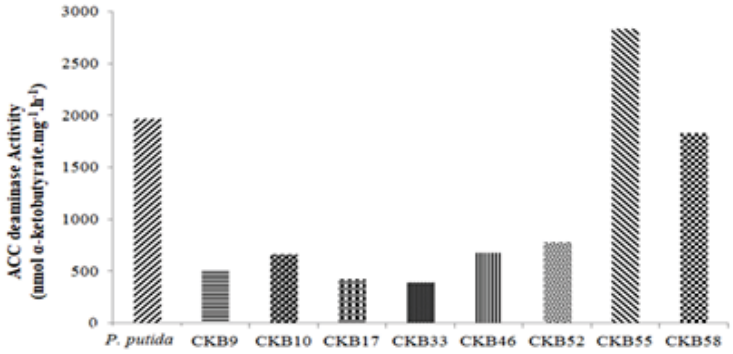

FIGURE 6
Quantitative ACC Deaminase Activities of Cultures

Considering the effects of salt concentrations on the growth of Gram-negative cultures which had ACC deaminase activity, 75% of the isolates were tolerant up to 1000 mM NaCl concentration. The highest amount of biomass was obtained from *Pseudomonas aeruginosa* CKB46 at 400 mM NaCl trial (7.4 mg/ml). It was found out that one culture has evolved up to 800 mM, which is *Serratia* sp. CKB33 (Figure 7). The growth of *Pseudomonas* sp. CKB9 was influenced as suppression at 400 mM salt concentration, although it was initially stimulated from low salt concentrations. On the other hand, growth of *Pseudomonas* sp. CKB10 was stimulated in all salt concentrations, and it was grown at 1000 mM salt concentration. Although the growth of *Enterobacter* sp. CKB17 was stimulated up to the 400 mM salt concentration, its growth was severely suppressed at 800 mM salt. The growth of *Serratia* sp. CKB33 was partially stimulated from the beginning, and this growth was continued up to 300 mM salt concentration, but was severely suppressed at 400 mM. *Pseudomonas aeruginosa* CKB46 is a culture with high adaptation to salt concentrations. The growth up to 400 mM salt concentration had increased in this culture, and the highest biomass was obtained from this bacterium at 400 mM salt concentration (7.4 mg/ml). Also, the culture was increased with high biomass at 800 mM (6.8 mg/ml), but it was suppressed at 1000 mM (4.5 mg/ml). Another tolerant culture is *Pseudomonas* sp. CKB52. The bacterium was partially stimulated up to 800 mM salt concentration, but it was suppressed at 1000 mM salt. Other cultures that can develop at 1000 mM salt concentrations are *Pseudomonas* sp. CKB55 and *Pseudomonas* sp. CKB58 (Figure 7).

As a result of the analyzes on the effect of salt concentrations on growth of the eight Gram-negative bacteria, five cultures for further studies have been selected. These cultures have a high salt tolerance. These cultures were incubated at their optimum conditions, then nitrogenase enzyme activity assays were carried out to investigate the biofertilizer potentials. At the end of study, the highest nitrogenase activity was obtained from *Pseudomonas* sp. CKB10 (808 nmol/mg.h). Furthermore, other cultures with high nitrogenase activity were *Pseudomonas* sp. CKB52 and *Pseudomonas* sp. CKB55. The lowest nitrogenase enzyme activity belongs to *Pseudomonas aeruginosa* CKB46 (Figure 8).

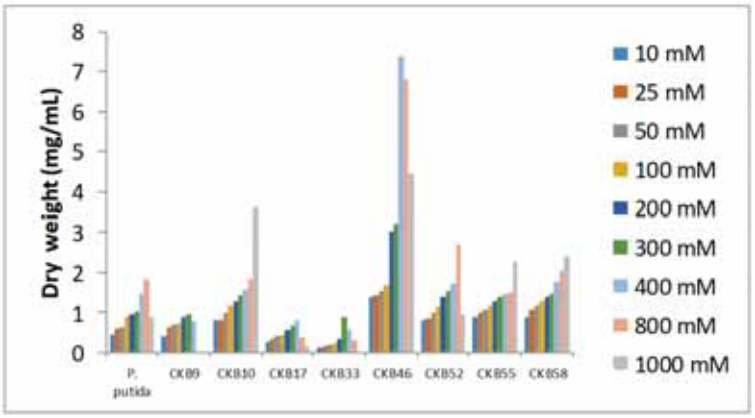

FIGURE 7
Effect of Salt Stress on the Growth of Cultures with ACC Deaminase Activity

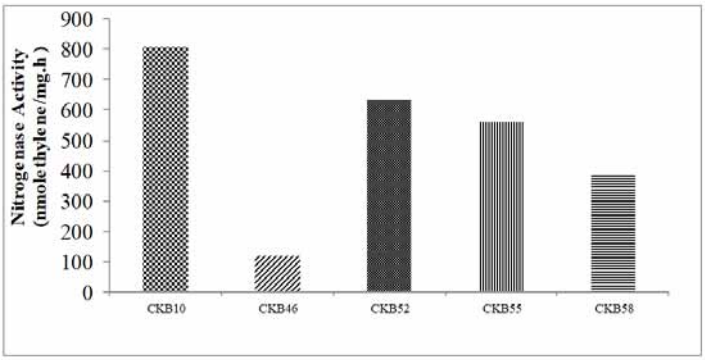

FIGURE 8
Nitrogenase Activities of Cultures

Five Gram-negative bacteria with high salt tolerance were investigated against five yield-spoilage pathogens for antagonistic activity. In this study, *Pseudomonas* sp. CKB52 had the highest antagonistic activity against *Erwinia caratovora* subsp. *caratovora* ECC100 (11 mm). The other high antagonistic activity belongs to *Pseudomonas* sp. This inhibition zone against *Agrobacterium vitis* was 10 mm. Only *Pseudomonas* sp. CKB58 isolate showed low activity against *Pseudomonas tomato* Pt52-a (3 mm). *Pseudomonas tomato* Pt52-a, which breaks the yield, has shown resistance to most cultures. The antagonistic activities of cultures against *Rathayibacter tritici* DSMZ7486 were found to be low. The highest antagonistic activity against *Rathayibacter iranicus* DSMZ7484 was found in *Pseudomonas* sp. CKB52 as 9 mm (Table 2). In another antibacterial activity study, standard antibiotics were used against yield pathogens. In this study, two antibiotics were used as a positive control, being penicillin and gentamicin. The inhibition zone of penicillin was determined against *Rathayibacter tritici* DSMZ7486 and *Rathayibacter iranicus* DSMZ7484, respectively as 28, 26 mm. As a result of the studies using the gentamicin antibiotic, the highest inhibition zone was found against *Erwinia caratovora* subsp. *caratovora* ECC100. This inhibition zone was 21 mm (Table 3).

TABLE 2
Antagonistic Activities of Bacterial Cultures with ACC Deaminase Activity Against Yield-Spoilage Pathogens

Bacteria	Inhibiton zone diameters (mm)				
	R. tritici DSMZ7486	*R. iranicus* DSMZ7484	*A. vitis*	*E. caratovora* ECC100	*P. tomato* Pt52-a
Pseudomonas sp. CKB10	4	-	10	5	-
Pseudomonas aeruginosa CKB46	4	-	3	4	-
Pseudomonas sp. CKB52	5	9	8	11	-
Pseudomonas sp. CKB55	2	2	-	-	-
Pseudomonas sp. CKB58	3	-	-	-	3

TABLE 3
Antibiotic resistance profiles of yield spoilage pathogens

Pathogens	Inhibiton zone diameters (mm)	
	P	GE
R. tritici DSMZ7486	28	nt
R. iranicus DSMZ7484	26	nt
A. vitis	nt	21
E. caratovora ECC100	nt	19
P. tomato Pt52-a	nt	19

P: Penicillin (10μg); GE: Gentamicin (10 μg); nt: not tested

DISCUSSIONS

The growth of plants and agricultural demands are severely affected from non-biotic stress. The regulation of ethylene levels in plants by bacterially produced ACC deaminase, is an important feature that can affect the physiology of the host plant. In soils with drought stress, drought-tolerant ACC deaminase-producing microorganisms can reduce stress on plants by reducing ethylene production, thus facilitating growth and development of plants [59]. Wang et al., [60] reported that soil microbial community structure varies under different tillage managements, especially in the 0-10 cm layer.

As a result of our studies, *Pseudomonas aeruginosa* CKB46 has showed an optimum growth at 45 ˚C (Figure 2), pH 7 (Figure 3), 200 rpm (Figure 4). Sivaprakasam et al. [61] reported that *Pseudomonas aeruginosa* has shown optimum growth at pH 7.5 and 160 rpm. In another study, it was reported that *Pseudomonas aeruginosa* UD-5 isolates showed optimum growth at 40 ˚C [62]. These results are similar with our work.

The highest ACC deaminase activity was determined from *Pseudomonas* sp. CKB55 (2833 nmol α-ketobutyrate.mg^{-1}.h^{-1}), and this value is higher than the value of *Pseudomonas putida* (Figure 6). Li et al. [63] have reported that *Pseudomonas* species have ACC deaminase activity. Ghyselinck et al. [64] stated that the ACC deaminase activity of *Pseudomonas* sp. R43582 was 310 nmol α-ketobutyrate.mg^{-1}.h^{-1}. In another study, ACC deaminase activity for *Pseudomonas* strains were given differently [55]. A similar study was conducted by Grichko and Glick [65]. In their study, *P. putida* ATCC17399 have ACC deaminase activity, and the value was 3.8 μmol.mg^{-1} protein.h^{-1}. In another research, the ACC deaminase activity of *Bacillus subtilis* HYT-12-1 was reported as 112 nmol α-ketobutyrate.mg^{-1}.h^{-1} [66]. The results obtained from our study were found to be higher than the data obtained from the literature, and the studies in the literature support our results.

As a result of our studies, *Pseudomonas aeruginosa* CKB46 was found to be tolerant to high salt concentrations (Figure 7). Different researchers reported that *Pseudomonas aeruginosa* was tolerant to high salt concentrations, too [61, 62]. These studies support our results.

As a result of nitrogenase activity studies, the highest nitrogenase activity was found at *Pseudomonas* sp. CKB10 (808 nmol/mg.h) (Figure 8). Han et al. [67] reported that the nitrogenase activity of *Pseudomonas stutzeri* was at 1400 nmol ethylene/mg protein/h. Borowiak et al. [68] reported that soil bacteria have various enzyme activities. Our findings are similar to these results.

The analysis of the antagonistic activities of cultures, isolated from wheat fields, that have ACC deaminase activity showed the following results. The highest antagonistic activity of *Pseudomonas* sp. CKB52 was found against *Erwinia caratovora* ECC 100 (11 mm) (Table 2). Weller [69] reported that fluorescent *Pseudomonas* strains were among the most effective root bacteria against soil pathogens. Algeblawi and Adam's study [70] reported that all of the bacteria (*Pseudomonas fluorescens, Bacillus thuringiensis, Bacillus subtilis*) showed antagonistic activity against *Erwinia caratovora*. In many studies, researchers reported that different bacteria shown antagonistic activity against various crop pathogens [71, 72].

CONCLUSIONS

Pseudomonas species are known to be microorganisms capable of metabolizing natural and synthetic organic compounds. These bacteria show a very wide spread at the plants and animals. They are valuable for plant growth because they have a lot of ecological diversity, have simple nutrient requirements and are capable of metabolizing a wide range of organic compounds. Therefore, it is thought that *Pseudomonas* will play an important role in the growth of plants in stressed soils.

As a result, *Pseudomonas aeruginosa* CKB46 had the highest tolerance to all NaCl concentrations and also showed other biological activities. We suggest that this bacterium can be used for sustainability in agriculture in all saline areas up to 1000 mM NaCl concentration. We believe that this bacterium contributes to sustainable agriculture because of its potential to reduce stress of the plant, its biofertilizer potential and its antagonistic activity against product pathogens. In future studies, it is necessary to analyze the growth of these bacteria, isolated from wheat fields under conditions of various organic pollutants, also to investigate the genome structures of isolates, with high activity and consequently, after the determination of gene expression of tolerant genera, studies are needed to transfer the gene of the plants.

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