Spatial and Temporal Variability of ACC Deaminase Activity in Saline Soils

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Project Objectives

1. Determine the population size and activity of bacteria that carry the ACC deaminase gene in avocado rhizosphere soil as affected by local variations in salinity across a transect of soils in the avocado production areas of Southern California.
2. Evaluate spatial variability in PGPR populations containing the ACC deaminase gene along the root axes.
3. Determine the minimum effective population size of PGPR with ACC deaminase activity in the rhizosphere that is necessary to reduce ACC concentrations in root tissues.

Approach and Procedures

This study was conducted to assess the functional diversity, distribution, and population dynamics of PGPR with ACC-deaminase activity that are associated with the rhizosphere of different avocado rootstocks for trees in soils having different levels of soil salinity. We also studied representative PGPR that could be cultured in the laboratory and their ability to increase plant growth when inoculated on to a model test plant. Our central hypothesis was that the roots of avocado trees in saline soils support growth of indigenous PGPR that can improve their tolerance to salinity stress. Understanding of the factors that affect the population size and activity of PGPR may thereby allow better management of the indigenous PGPR through cultural practices or by soil inoculation with superior PGPR having multifunctional traits.

To study the PGPR associated with avocado trees, rhizosphere soil samples were collected from 3 locations in a commercial avocado orchard near San Juan Capistrano, CA. The soil chemical properties were determined using ASA Methods for Soil Analysis (ASA, 1982). The salinity levels in the samples were measured using 1:2 soil water extracts and a portable conductivity meter. Each location contained 10 - 12 year-old Hass avocado trees that had been grafted on to different rootstocks including clonal rootstocks of Dusa, Toro Canyon, and Duke 7 that are relatively salt tolerant (Crowley and Arpaia, 2010). At each location, rhizosphere samples were taken from 2 trees on clonal rootstocks and from one tree produced on the Mexican rootstock. Samples were taken at 0-10 and 10-20 cm depths, after which they were placed in sterile polyethylene bags, stored on ice, and transported to the laboratory for microbiological analyses.

Fine feeder roots (< 2mm diameter) from the avocado trees were separated from the bulk soil, after which PGPR bacteria associated with the roots were isolated by culture based methods using DF minimal salts (Dworkin and Foster, 1958) containing ACC (3 mmol L^{-1}) as a sole source of nitrogen, and incubated at 28 C for 48 h. A root print study also was conducted to identify the root zones where the highest population densities of PGPR occurred. Small pieces of

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fine (< 1 mm) and coarse roots (2-3 mm) were placed on DF agar medium containing ACC. The plates were incubated at 28±1°C for 24 h and were examined for microbial growth after 48 h.

Approximately 100 colonies from 20 representative strains were selected for characterization. The isolates were identified using 16S rRNA gene sequencing, and were characterized for ACC-deaminase activity, phosphate solubilization, siderophore production, IAA and cyanide production. The ACC-deaminase activities of the bacterial isolates were determined according to Penrose and Glick (2003). Auxin production by the rhizobacteria was measured in assay both with and without L-tryptophan (L-TRP). All auxin measurements were performed in triplicate. The same procedures were performed for control samples to determine possible auxin production in the absence of L-TRP. Qualitative measurements of phosphorus solubilization by individual isolates were determined using National Botanical Research Institute (NBRI) growth medium (Mehta and Nautiyal, 2001). Siderophore production was assayed according to the universal method of Schwyn and Neilands (1987). Other tests were performed for qualitative determination of siderophores (Jalal and van der Dick, 1990) and cyanide production. All experiments were performed with 3 replicates for each bacterial strain.

The data for colony forming units in the rhizosphere of different root stocks were subjected to Analysis of Variance. The means were compared using Tukey's test or by Least Significant Difference (LSD) test; statistical significance was evaluated at p= 0.05. The correlation between soil salinity and population of ACC-deaminase bacteria was also calculated. All of the statistical analyses were conducted using Minitab 16 software (Minitab TM Inc. USA). The means and standard errors were calculated using Microsoft Excel 2007.

Results

The rhizosphere bacterial communities of all 4 rootstocks had relatively large population densities of culturable ACC-deaminase bacteria that ranged from 1.5× - 8.5× cfu g^{-1} root tissue (Table 1). Generally, there were no significant differences in bacterial population densities for soils collected from different locations and depths, although trends suggested that populations were larger in saline soils. PGPR population densities were statistically significant at one location where trees on the Mexican and Dusa rootstocks were interplanted. A positive significant correlation (R2= 0.94**) between soil salinity and ACC degrading rhizobacterial populations was observed for root samples collected from the Mexican rootstocks.

A root printing study was conducted to qualitatively identify the locations where the highest population densities of ACC-degrading bacteria occurred in the plant rhizosphere. Results using this method showed that the greatest population densities occurred near the root apices (root tip and zone of elongation) or at the sites of lateral root emergence (Fig 1). Minimum bacterial populations were observed on the agar prints generated from coarse roots.

Among the 20 isolates that were evaluated for their PGPR traits, 10 were identified as *Pseudomonas*, four as *Variovorax*, two as *Bacillus* and one each belong to *Burkholderia*, *Agrobacterium*, *Chryseobacterium* and *Stenotrophomonas* (Table 3). This limited census suggests that Pseudomonas spp. were the dominant culturable PGPR in the avocado rhizosphere.

Table 1. *Soil salinity and population of PGPR with ACC deaminase activity associated with clonal saline tolerant (Dusa and Toro Canyon) and nonclonal saline intolerant (Mexican) avocado rootstocks at three locations in Southern California*

Rootstock and Tree #	Soil salinity (dS m^{-1})	Colony forming units (cfu) in rhizosphere soil associated with roots at two soil depths	
		1-10 cm	10-20 cm
Site-I			
Mexican I	3.9±0.08	8.5 ×	2.7 ×
Dusa 1	1.9±0.05	1.7 ×	3.9 ×
Dusa 2	2.3±0.03	5.3 ×	1.7 ×
Site-II			
Mexican 2	1.8±0.07	5.5 ×	3.7 ×
Toro Cnyn 1	1.5±0.08	1.9 ×	1.6 ×
Toro Cnyn 2	0.9±0.06	7.0 ×	3.1 ×
Site-III			
Mexican 3	3.5±0.08	5.0 ×	1.5 ×
Duke 1	3.4±0.08	3.3 ×	5.1 ×
Duke 2	1.7±0.02	1.2 ×	5.4 ×
Least sig. diff.	-	209	286

In addition to ACC degradation, several isolates expressed additional traits that are commonly associated with plant growth promotion (Table 4). Approximately half of the isolates were positive for phosphate solubilization, 17 for siderophore production and 9 produced cyanide. All 20 strains had the ability to produce IAA when supplemented with L-tryptophan (L-TRP). Large differences were observed with respect to IAA production (Table 4). None of the isolates were able to produce IAA in the absence of L-tryptophan. The greatest amount of IAA production was observed for *A. tumefaciens*, which was followed by *P. lurida*, and *B. licheniformis*.

Quantitative measurements of ACC-deaminase activity showed that all of the isolates produced α-ketoglutarate (the degradation product of ACC) when grown in liquid media containing ACC as a nitrogen source, but that the rates varied substantially among the different strains (Table 2). Rhizobacterial isolates that had the highest ACC-deaminase activities were associated with the Toro Canyon and Dusa rootstocks, although this could not be statistically evaluated using the experimental design in the present study. The highest ACC-deaminase activity was observed for *V. paradoxus*, which was isolated from roots produced by the Dusa rootstock.

Table 2. *Characterization of selected rhizobacterial isolates associated with different root-stocks of avocado growing in Southern California.*

Isolate	Rootstock	ACCD activity µmol product mg^{-1} protein h^{-1}	IAA production mg kg^{-1}	Hydrogen cyanide production
Pseudomonas trivialis	Mexican	8.43	2.76	+
Bacillus megaterium	Mexican	23.11	33.71	-
Burkholderia sp.	Dusa	15.17	12.41	-
Variovorax paradoxus	Dusa	185.51a	3.27	-
Variovorax paradoxus	Dusa	175.23a	9.00	+
Bacillus licheniformis	Dusa	44.56	39.11	-
Chryseobacterium sp	Mexican	8.00	5.07	-
Pseudomonas lurida	Mexican	58.26	43.23	+
Pseudomonas sp.	Mexican	4.91	13.42	-
Pseudomonas sp.	Toro canyon	113.12a	11.21	-
Agrobacterium tumefaciens	Toro canyon	15.69	59.44	-
Variovorax paradoxus	Toro canyon	127.53a	5.63	-
Pseudomonas putida	Toro canyon	75.28	16.55	+
Pseudomonas fluorescens	Toro canyon	113.79a	7.82	-
Variovorax paradoxus EPS	Mexican	104.55	2.31	+
Pseudomonas trivialis	Mexican	7.57	18.05	+
Stenotrophomonas sp	Duke	18.24	30.33	-
Pseudomonas sp.	Duke	27.64	32.75	+
Pseudomonas. chlororaphis	Duke	6.40	1.09	++
Pseudomonas azotoformans	Duke	46.19	10.89	+

With regard to other PGPR traits, all of the *Bacillus* isolates were positive for phosphate solubilization. Sixty percent of the *Pseudomonas* and 25% of the *Variovorax* isolates could solubilize phosphate. The maximum halo size was recorded with *Burkholderia* sp. followed by one of the pseudomonads. The other *Pseudomonas* spp. showed high variability in their rate of phosphate solubilization. Most of the PGPR produced siderophores, including 90% of the *Pseudomonas* spp. and 75% of the *Variovorax* spp. (Table 5). The *Bacillus, Burkholderia* and *Agrobacterium* isolates also were positive for siderophore production. Assays examining cyanide production showed that 9 of the isolates produced cyanide (Table 4), most of which were *Pseudomonas* spp. Seventy percent of the pseudomonads and 50 percent of the *Variovorax* were able to produce cyanide (Table 5). *Bacillus* sp. also was positive for this trait.

Discussion

As the availability of high quality water for agriculture becomes increasingly uncertain, farmers in California and other semi-arid regions have become very interested in biotechnology that can be used to improve plant tolerance to drought and salinity. One method that has received a lot of attention, especially in developing countries has been the development of biofertiizers or soil

inoculants that can be used to improve plant growth under saline conditions. There are now hundreds of reports that have identified specific bacteria that can be used to inoculate plants, but one of the greatest problems has been lack of consistency and difficulty in determining the extent to which soil inoculants actually improve plant growth, or if they are even necessary. As all soils contain indigenous populations of PGPR, more information is needed on how soils might be better managed to improve the population densities and activity of those strains that are already present in the soil. This is especially relevant for orchard crops such as avocado that have root systems that are strongly affected by problems with salinity and that require careful water, nutrient, and organic matter management to obtain good crop yields.

Plant growth promoting rhizosphere (PGPR) bacteria that carry the ACC (aminocyclopropane carboxylate) deaminase gene have been shown to greatly improve the salinity and drought tolerance of plants by lowering stress-ethylene concentrations in the root tissues (see review Glick et al., 2007). While diverse soil bacteria carry this trait, very little is known yet about the distribution of this gene in different soils, or the extent to which gene expression and activity is affected by soil physical and chemical properties. The results of the present study supported our hypothesis that avocado roots maintain relatively high populations of culturable ACC-deaminase rhizobacteria in soils having a wide range of salinity levels. The survey also suggested that the abundance of PGPR varied with location and rootstock (Table 2). For trees on the same rootstock, the populations of ACC-degraders tended to be slightly greater in soils having higher salinity levels, which might be due to greater availability of ACC, which is synthesized in response to salinity stress (Lutts et al., 1996; Bar et al., 1998). This finding was also supported by root imprints on agar, which showed that population densities were much higher in the zone of elongation of young roots than that found on older coarse roots, and that would be expected to accompany the relatively greater production of ACC in the elongation zone of young roots (Arshad and Frankenberger, 2002).

We were also interested in determining whether rootstocks that are salt tolerant might harbor higher population densities or select for bacteria with superior ACC degrading abilities. This hypothesis was partially supported by our observation that the most efficient ACC-degrading rhizobacteria were associated with the rootstocks of Toro Canyon and Dusa (Table 4), which are relatively more tolerant to salt stress than the Duke 7 and Mexican rootstocks (Crowley and Arpaia, 2010). However, further investigation with greater numbers of replicates and soils will be necessary to confirm whether such a relationship actually exists and the extent to which the population and activity of ACC degrading bacteria varies for these rootstocks at other locations and soil types.

The role of PGPR containing ACC-deaminase for improving the salinity tolerance of plants has been reported by many researchers in studies where seeds or plants were inoculated with PGPR expressing ACC-deaminase activity (Mayak et al., 2004; Saravanakumar and Samiyappan, 2006; Ji et al., 2006; Cheng et al. 2007; Jalili et al., 2009; Siddikee et al., 2010). Members of our group have also documented the effectiveness of inoculation with ACC deaminase PGPR in eliminating the negative impacts of salinity on growth and yield of different crops, under both axenic and natural conditions (Nadeem et al., 2007, 2009; Kousar et al., 2009; Zahir et al., 2009). However, the diversity of PGPR, and the relationships between population size and activity and plant growth responses still remain poorly understood.

Phylogenetic analysis of the selected isolates showed that PGPR associated with avocado roots were comprised of mixtures of species from several genera. Among these, the dominant culturable PGPR included species of *Pseudomonas, Bacillus* and *Variovorax*. Other researchers

have shown that these genera are common in the rhizospheres of a variety of plants (Aeron, et al., 2010; Timmusk et al., 2011; Sharma et al., 2011). In our survey, there was large variation among isolates of these species with respect to the different combinations of growth promoting traits that they carried. All of the isolates were able to produce IAA, but with variable efficiency. Interestingly, all the ACC degraders were able to produce IAA only in the presence of L-TRP which may imply that IAA synthesis by the PGPR is dependent on the amount of L-TRP present in root exudates. About half of the isolates identified in the present research were able to solubilize phosphorus, 85% were able to produce siderophores, and some were able to produce cyanide. Other researchers have also reported that indigenous rhizobacteria possess a variety of plant growth promoting traits, alone or in combination, including ACC-deaminase activity, IAA production, phosphorus solubilization and siderophore and cyanide production (Timmusk et al., 2011; Sharma et al., 2011).

The results of present study provide an initial insight into the relationship of ACC-deaminase PGPR and soil salinity tolerance of salt sensitive tree crops such as avocado. A better understanding about the relationship between the population densities, and activities of PGPR in the rhizosphere, could help in optimizing management practices to maintain effective PGPR populations that can improve root growth under saline conditions, thereby improving nutrient uptake, water use efficiency, and ultimately crop yields.

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