Cultivable bacteriome dynamics in different Persian oak tissues and soil during Oak Decline Syndrome development in Iran

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Abstract

Persian oak decline is a syndrome within the oak decline complex in Iran. Profuse stem bleed-ing and larval galleries of the native buprestid, Agrilus hastulifer characterize the disease. A systematic study comparing healthy with diseased trees was undertaken. This work reports the result of isolations from healthy trees and diseased tissue in affected trees, at eight sites in Iran. Culturable bacterial communities were identified using the 16S rDNA sequencing. A significantly higher proportion of symptomatic tissue pieces from diseased trees (Disease In-dex=5) yielded bacterial growth than other disease indexes (83.78%). Significantly higher yields were also obtained from bulk and rhizosphere compared with the root, leaf, and stem. Overall bacterial communities compositions varied at each site, but significant similarities were evident in diseased tissues at all sites. Enterobacteriaceae were dominated in diseased trees whereas Bacilluceae and Moraxellaceae were remarkable more abundant in healthy trees. Sig-nificant associations occurred between diseased tissues and certain bacterial species, implying that the cause of tissue necrosis was not due to random microbiota. Brenneria goodwinii, Ser-ratia marecescence, and Dickeya chrysanthemi were key species consistently isolated from diseased tissue; Campylobacter jejuni and an un-named Clostridium taxon were also frequent-ly isolated from both healthy and diseased trees. It was concluded that there was a shift in the cultivatable bacterial microbiome of diseased trees, with Enterobacteriaceae strongly repre-sented in symptomatic but not healthy tissues. No single dominated species was isolated from diseased tissues, so tissue degradation in oak likely have a polymicrobial cause.

INTRODUCTION

Worldwide, the healthy and sustainability of oak forests has been threatened by oak decline (Gibbs & Greig, 1997; Denman et al., 2018) that manifests itself by the progressive decrease in tree vigor without any clear evidence of a single causal factor (Ciesla & Donaubauer, 1994). This is an extremely important problem because oak species (*Quercus* spp.) are dominant in most of the hardwood forests of the Northern Hemisphere (Harlow & Harrar, 1969). Since the 2009, oak decline on native oak species, *Quercus brantii* L. has been a chronic problem throughout the Zagros forest in Iran (Ahmadi et al. 2014). The Zagros forests, with an area of around 5 million ha, account for almost 40% of the country's forests (Sagheb-Talebi et al. , 2013). These forests are one of the most important & sensitive ecosystems in Iran and provide a home and livelihood for approximately 10% of Iran's population (DoE/GOIRI, 2004). (Salehi & Karltun., 2010; Mashayekhi et al., 2010). In this region, where oak forests occupy over 90% of forested land, they are extremely valuable economically and ecologically (Panahi et al., 2012; Salehi & Karltun., 2010). Oak trees are a "keystone" to biological diversity because their mast production plays a vital role in the food webs of the forest (Purhashemi et al., 2004). Decline in many cases led to a destruction of oak trees in Zagros region especially in central and southern Zagros somewhere eminent decline phenomena have been increasing in Ilam, Fars, Kohkilloieh, Lorestan provinces (Sepahvand & Zandebasiri, 2014). Quantitative studies on the number of trees affected have not been carried out, but estimates from the Forests, Range and Watershed

Management Organization suggest that thousands of trees are affected, and on monitored sites more than 80% of symptomatic trees destroy every year (Ahmadi et al. 2014). The cause of decline attributable to multiple factors include biotic and abiotic stressors (Brasier, 1996; Sicoli et al., 2002; Moreira & Martins, 2005; Desprez-Loustau et al., 2006). In recent decade, climatic parameters (dust storm, reduced rainfall, higher temperatures and longer drought period), some human activities such as land-use change, diseases as charcoal disease and insect holes have occurred and these are considered to be the causes of decline in the Zagros forests of Iran (Khalyani & Mayer, 2013; Mirabolfathy et al., 2013; Zandebasiri et al., 2017). Affected trees are characterized by symptoms of bark cracking black necrotic lesions and stem and twig blight, bacterial ooze, unclear blackish discolorations, and the presence of larval galleries of wood-eating pests (Taghimollaei & Karamshahi, 2017; Jozeyan & Abaii, 2011). Declining oak trees with symptoms similar to those have been reported in Britain and continental Europe (Brady t al., 2010; Brown et al., 2015; Denman, 2014; Falck, 1918, ; Gibbs, J., Greig, 1997; Jacquiot, 1950) where the disease was mostly attributed to oak borer attack, but some reports implicated a microbial component (Vansteenkiste et al., 2004; Thomas, 2008; Biosca et al ., 2003; Denman & Webber 2009; Denman et al., 2012; Denman et al., 2014; Denman et al., 2016; Brown et al., 2016; Brown et al., 2017; Reed, 2016; Sallé et al., 2014). Until now, number of oak pathogens have been identified worldwide and the almost of them are of fungal etiology. Specially, some canker-causing agents, such as *Biscogniauxia mediterranea* , *Diplodia corticola* and *Discula quercina*(Cooke) Sacc. have constantly been found to be associated with declining oak trees, and their pathogenicity to Mediterranean oak species has been exhibited (Luque et al., 2000; Linaldeddu et al., 2009; Inacio et al., 2011; Maddau et al., 2011). In addition, oak death, caused by the non-native invasive pathogen *Phytophthora ramorum* , is an emerging forest disease that has destroying tens of thousands of trees in 12 coastal counties of central and northern California (Rizzo & Garbelotto, 2003, Brown & Allen-Diaz, 2009).

Recent studies on oak decline in Iran have showed that the fungus*B. mediterranea* and *Truncatella angustata* are commonly associated with the decline. This fungus has been reported to be aggressive on drought stressed hosts (Mirabolfathy et al., 2013; Alidadi et al., 2018). Remarkable, bacterial diseases of oak have not so well studied, probably as their impact on the oak forests has been less important than other factors, and also due to difficulty of identification and characterization of the bacterial causative factors and their mechanisms of action (Kovaleva et al., 2015). Previously, several bacterial pathogens have been reported as causative agents of oak decline that they play a key role in development of AOD (Barnard et al., 1998; Scortichini et al., 1993; Brady et al., 2010; Brady et al., 2012; Denman et al., 2012). Until now, two new genera and thirteen novel species detected (Denman et al., 2012; Brady et al., 2010; Brady et al., 2014). Recently, studies have been done on identify pathogenic bacteria associate with oak decline in Iran and only two reports have been published in this field. In these studies have been reported*Brenneria* spp. and *Rahnella victoriana*(Moradi-Amirabad et al., 2019) and *Bacillus pumilus* and*Stenotrophomonas maltophilia* (Ahmadi et al., 2019) as potentially causative agents involved in Persian oak decline in Zagros forests. As for the importance of fungi and bacteria as biotic factors in the phenomenon of oak decline and the lack of information in this regard, needed to further research on the effects of bacteria in this phenomenon. To gain insights, and select putative causal agents for the oak decline pathosystem and also to understand the community structure and diversity of plant associated endophytic bacterial communities and their relationships in diseased and healthy tissues a systematic study examining statistical associations of microbial species with healthy and diseased trees was required. A number of approaches were taken to obtain an overview and comparison of the composition of the microbiome, these included metagenomics (unpublished results). However, conventional isolation methods were applied in this study, as the main purpose of the current study was to obtain cultures to further study the biology, phenotype and genomic aspects so that appropriate selections for Koch's postulates testing could be made. The hypothesis behind this study states that there are similarities in the composition of the cultivatable microbiomes of the diseased tissues in oak decline symptomatic trees vs healthy trees. The aims of the study were to: isolate bacteria from the stems, root, leaf and soil of healthy and diseased native oak trees in the Iran, determine similarities and differences in the composition of bacterial communities at the site level, as well as at existing at different stages of disease (healthy vs diseased) and at the sample type levels.

2. MATERIAL AND METHOD

2.1. Study site, investigation of symptom and sample collection

Field survey were selected from a remarkable area of Zagros forests during 2015-2016. This area is located in Ilam province that declined 80% of oak trees (Ilam covers 640000 ha of these forests) (33deg38'15"N 46deg25'22"E). Eight counties were selected based on representative spatial distribution of symptomatic. Knowledge of the distribution of symptomatic in the western Iran was obtained during a previous Forests, Range and Watershed Management Organization project on the oak decline in Iran (Forests, Range and Watershed Management Organization, 2013). The sites chosen were: Arghavan (N33 39.709 E46 27.065), Saleh Abad (N33 32.458 E46 15.002), Tange Dalab (N33 41.935 E46 25.078), Chogha Sabz (N33 35.775 E46 26.497), Malek Shahi (N33 29.615 E46 29.700), Chavar (N33 41.120 E46 06.091) Eyvan (N33 46.148 E46 22.203) Gale Jar (N33 43.122 E46 20.160). Development of the symptoms was evaluated using a decline index (DI) with five categories from 1 to 5, depending on the symptoms and the length of the lesion or dead parts compared to the shoot length. Descriptions of symptoms were based on naturally infected materials. The decline indexes were as following: DI 1: 1-10% damage, DI 2: 10-30% damage, DI 3: 30-60% damage, DI 4: 60-90%, and DI 5: with 90-100% damage (Gonzalez Alonso, 2009; Ishihara et al., 2015). Different sample from three trees from each DI in each eight representative sites were randomly collected. In total 120 symptomatic and asymptomatic trees were sampled (n= 120 trees). The symptomatic tissues consisted of lesions area at the advancing margins of the lesions, wounds around knots, resinosis region and wilting, yellowing of leaves. Sampling was not performed from larval galleries, However, during the samplings the decline symptoms and situation and form of larval galleries were evaluated visually. To isolate bacterial populations, three different tissues, including stem (sapwood and heartwood), leaf and root, and soil (bulk and rhizosphere) were sampled from each symptomatic and asymptomatic tree. Root segments were collected from buttress and feeder roots in the stem base at a depth of 5 to 15 cm. Stem, root and leaf samples were excised and placed in the plastic bags. Sampling strategy from soil consisted of taking randomized soil samples from four areas of the plot (horizontal sampling) and at the depth of 15 cm below the soil surface. Rhizospheric soil samples were taken at 10 cm depth from the soil surface. Soil was homogenized manually by thorough physical mixing. The collected samples were placed into fresh unused polythene bags, kept cold (4 degC) and delivered to the lab within 24 h.

2.2. Sample taking and preparation for isolation

The stem (sapwood and heartwood), root and leaf samples were taken and prepared as previously described by (Biosca et al., 2003; Sapp et al., 2016). The samples were collected using sterile spades, clipper and gloves. The spades and clipper were sterilized between different individual plants. The roots were shaken vigorously to separate them from loose soil. Stem, root and leaf samples (approximately 1 cm in length) were surface disinfected in 80% ethanol for 15 s, followed by 2% sodium hypochlorite solution for 1–2 min, and rinsed three times in sterile distilled water. Tissue pieces were air dried for 30 min and then for bacteria isolation, 1.9 g of sampled materials was crushed in 20 ml of sterile water using sterile mortar and pestle (Trivedi et al., 2011). About 100 µl of the resulting suspension was poured and spread uniformly onto several culture plates containing nutrient agar medium (NA), composed of: 5 g beef extract, 10 g peptone, 5 g NaCl, 15 g agar and 1 l distilled water, pH 7.0 (Jin et al., 2014; Schaad et al., 2001; Ishihara et al., 2015). Using a sterile mortar, the soil samples was homogenized, weighted and distributed in ten Eppendorf sterile microtubes (one gram in each microtube). In order to resuscitate the bacterial species, present in soil, all ten aliquots were hydrated with 100 ml sterile peptone water, magnetically homogenized and incubated at 32°C for 2 hours. After the incubation period from every aliquot tenfold dilutions were done (1 ml initial suspension + 9 ml sterile peptone water). Because a high bacterial contamination was expected, for every aliquot of soil sample six tenfold dilutions were performed. The sixth and the fifth dilution from every aliquot was then entirely filtrated through 0.45 µm (Merck-Millipore®) hydrophilic filter, and the filters were inoculated at the surface of the Nutrient Agar (Merck®) solid culture medium and incubated at 32°C (Constantin et al., 2016).

Isolations were incubated under aerobic conditions at room temperature for two to three weeks, but examined

every 2–3 days, and when present, bacteria were sub-cultured by transferring to nutrient agar (NA). Bacterial subcultures were later streaked onto nutrient agar and processed to obtain single colony cultures (Lelliot & Stead, 1987). Single bacterial colonies were checked for purity and grouped according to colony morphology, cell shape, growth rate and Gram stain. And also Biochemical characteristics of the bacterial colonies, including oxidative/fermentative test (O/F), spore production, protease, oxidase and catalase production; presence of fluorescent pigment; nitrate reduction; fermentation with production of acid or acid and gas of galactose, glucose, lactose, maltose, rhamnose, sucrose, fructose, xylose, glycerol, mannitol and sorbitol; pectin digestion using a sodium polypectate were tested Bergey's manual of determinative bacteriology (Bergey et al., 1939) for screening of large numbers of isolated bacteria and similar identities from the same chip of tissue were removed.

2.3. DNA extraction, PCR amplification and fungal and bacteria diversity analysis

Bacteria strains identification from single colonies were transferred to 5 ml of LB broth using either a sterile pipette tip or inoculating loop. Cultures were incubated in a shaking (or stationary) incubator (28–30 *C, 250 rpm) overnight (>16 h). The SDS-based method was used for DNA extraction from bacteria according to a modified version of the protocol previously developed by Zhou et al. (1996). The primers 27F - 1541R were used for amplification of 16S rDNA gene (Embarcadero-Jimenez et al., 2014). The reaction mix consisted of 1 µl of the DNA as the template, 10 pmoles of each primer, 0.2 U Taq polymerase (Thermo Fisher Scientific, USA), 5 µL of 10X Taq buffer [10X buffer composition: Tris-HCl pH 9.0; PCR enhancers; Kcl; 20 mM MgCl2] and 10 mM dNTP mix (Thermo Fisher Scientific, USA). The final mixture was adjusted to 30 µl by addition of sterile and purified water. The amplification steps included initial denaturation at 95°C for 7 minutes, 35 cycles of denaturation at 95°C for 50 s, annealing at 56°C for 1 min and extension at 72°C for 50 s with the final extension of 72°C for 5 min. PCR products were separated by electrophoresis on a 1% agarose gel in TAE buffer and visualized using ethidium bromide staining on a UV transilluminator. The PCR product was purified with a QIAquick PCR purification kit (Qiagen Inc., Chatsworth, CA, USA). Sequencing of the purified 16S rDNA was performed using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, U.S.A) as recommended by the manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer.

2.4. Data processing and statistical analysis of data

Bacterial growth from tissue pieces on agar was recorded. Differences in percentage yield (calculated as pieces of tissue having bacterial growth, out of pieces plated), between healthy and symptomatic tissues were tested using a Generalized Linear Model with binomial error distribution and logit link function (SPSS 20.0, IBM North America, New York, USA) (Denman et al., 2016). Sequences were identified using the BLAST option at NCBI GenBank. Sequences with >97% similarity were assigned to the same species. Variability in the endophyte assemblages in the eight site, at different stages of development disease (disease index) and in different parts of plants (roots vs. aerial organs and soil) was measured using permutational analysis of variance (PERMANOVA, 9999 permutations, use of Bray-Curtis dissimilarities) with the package vegan (Oksanen et al., 2013). A multidimensional scaling (MDS) analysis was performed to illustrate plot similarity in terms of bacteria species composition (using the metaMDS function (Oksanen et al., 2016). MDS analysis is an ordination technique aiming at iteratively collapsing multidimensional information (in this case, plot species composition) into an optimal lower number of dimensions while conserving the rank order of distances. Points that are closer together on the plot are more alike than those further apart in species compositions (Minchin, 1987). Analysis of similarities (ANOSIM) was performed based on 999 permutations to test whether bacterial communities were significantly different among sample type or sampleing sites. In order to incorporate taxonomic data, we used the direct-gradient ordination technique, Canonical Correspondence Analysis (CCA), which concurrently showed pond taxa (Hall & Smol, 1992). This kind of ordination is appropriate when assessing community dynamics because it does not use Euclidean based metrics that assume linear trends in community change. Not only does the ordination show environmental factors influencing community change, but results suggest potential interactions between taxa (Amaral-Zettler et al., 2010).

The first two axes, CCA1 and CCA2, typically account for the majority of observed variation. All axes are constrained to present a linear combination of the ecological factors that maximizes the dispersion of taxa (Hall & Smol, 1992). The vegan package was used for MDS and CCA analyses.

3.RESULTS

3.1. Identification and phylogenetic analyses of endophytic bacteria

The composition of bacterial communities associated with oak tissues from trees with and without disease symptoms at eight sites was studied. Totally, 120 trees were studied from which 360 tissue samples and 240 soil samples were processed. After isolation a total of 3 % of the bacterial colonies failed to grow during the subculturing process or could not be cultured following storage at –80 degC; these bacterial colonies were removed from further analysis. Thus, a final total of 948 colonies were studied. From the 948 endophytes that colonized fragments, 386 were randomly isolated and grouped according to their morphological characteristics and biochemical test. From grouped colonies 148 endophytes, from each morphogroup were randomly selected and purified for DNA extraction. Analysis of variance of 600 sample indicated that the number of Recovered isolates were varied significantly by samples type ($F= 10.42$, $P<0.05$) and disease indexes ($F= 8.99$, $P<0.05$). A significantly higher proportion of symptomatic tissue pieces from diseased trees (Disease Index=5) (83.78%) yielded bacterial growth than other disease index while 14% of samples of healthy tree with DI=1 and DI=2 yielded bacteria. Significantly higher yields were also obtained from bulk and rhizosphere compared with the root, leaf and stem ($p < 0.01$) with root sample giving intermediate results (Figure 1).

A total 148 obtained bacteria isolates were identified using 16S rRNA sequence and biochemical data (Table 1) and were classified in 31 bacterial species or taxa, but there was variation in consistency of occurrence. Generally, there were few dominant species ([?]50 isolates over the whole study), namely *Stenotrophomonas maltophilia* ,*Pantoea agglomerans* , *Campylobacter jejuni* ,*Achromobacter xylosoxidans* , *Serratia marecescence* and*Dickeya chrysanthemi* and many rare or singletons species (Table 1). The main bacterial families present in oak included:*Alcaligenaceae, Bacillaceae, Brevibacteriaceae, Campylobacteraceae, Clostridiaceae, Microbacteriaceae, Enterobacteriaceae, Moraxellaceae, Paenibacilliaceae, Pseudomonadaceae, Pectobacteriaceae* and *Xanthomonadaceae* . In terms of familly, most isolates belonged to *Bacillaceae* (24.32% of the total number of isolates), followed by *Enterobacteriaceae* (21.62%) and lastly *Xanthomonadaceae* (10.13%). Isolates from family*Brevibacteriaceae* comprised only 1.35% of the total and only two isolates were found from of the genus *Brevibacterium* (Table 1, Figure 2).

Composition of the bacterial isolates by site is shown in Figure 3 according to the family. Identified families as *Bacillaceae* were observed in all sites. The relative differences in the proportions of the *Bacillaceae* and *Enterobacteriaceae* families were observed between the eight sample sites. *Brevibacteriaceae* were isolated only in the Saleh Abad area and also *Pseudomonadaceae*were exhibited only in the Malek Shahi region. *Bacillaceae* family in Arghavan and *Enterobacteriaceae* family in Chogha Sabz sites exhibited differences in the proportion of isolates in compared to isolates in the other sites (Figure 3).

The relative composition of the bacterial isolates by disease index is shown in Figure 4 according to family. The most variation in composition of the bacterial family observed in DI= 3 and followed by DI=4. Among the identified families, *Bacillaceae* were observed in all disease indexs. *Moraxellaceae* were isolated only in the DI=1 and also*Pectobacteriaceae* were discovered only in the DI=4. Notable point is the most *Bacillus* species were in low disease index, that's mean in primary stages disease (indexes 1 and 2) situations, usually the*Bacillus* species were dominant. In contrary,*Enterobacteriaceae* family were dominant in disease tree (indexs 4 and 5). The ratio of *Brevibacteriaceae* were the same in both DI=3 and DI=4 (Figure 4).

3.2. Distribution Patterns of Plants Microbiotes

3.2.1. Ordination of endophytic prokaryotic communities

We applied permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis distance matrix with 999 permutations to test the influence of the eight different sites, at different stages of disease development (Disease Index) and in different parts of plants (roots vs. aerial organs and soil) on the

bacterial communities. PERMANOVA analysis showed a significant effect of the different sites on bacterial assemblages (p-value 0.001, F = 4.4769) and also bacteria assemblages were significantly affected by disease index (p-value = 0.001, F = 5.1034) but there were no significant differences between different samples (p-value = 0.24, F = 1.29). Moreover, bacterial community composition were significantly affected by the interaction between disease index and site (p-value = 0.036, F = 1.6750). There were no significant differences between sample type and disease index (p-value = 0.395, F = 1.0904). (Table 2). PERMANOVA analysis yielded more sensitive results in all four displayed statistics and principally confirmed results from ordinations.

In the two dimensions of the MDS plot (Fig 5), bacteria species composition was markedly different in Tange Dalab and Gale Jar sites compared with other sites. Tange Dalab site had the most similar bacteria species composition to Chogha Sabz site. Bacteria species composition was highly variable among Chavar, Malek shahi, Eyvan, and Arghavan (fig 5a). Variations between samples from different disease index (DI) as illustrated in Figure 5b. DI 5 and DI 1 respectively, showed the strongest difference from other DIs. However, some community similarities were observed in samples from DI 3 and 4. In contrast, points by sample type were mixed on the plots. This suggests no difference in species composition between the different sample types (fig 5c). The overall separation was confirmed by ANOSIM. Bacterial communities were significantly different among sites (ANOSIM R=0.08416, P = 0.004) and disease indexs (ANOSIM R=0.04968, p= 0.025). But there were no significant differences in sample types (ANOSIM R=-0.00119, P =0.496).

Using CCA, the most significant ecological variables which correlated with fluctuations in bacteria taxa were plotted in Figure 6. Taxa and samples were correlated to different sites, different disease indexes, and varius sample types (vectors in Figure 6). In the analysis of the relationship among the composition of the bacteria community and the ecological factors, we observed that the environmental variables (site, disease index, sample type) affected the structure of the bacteria community (Fig. 6). The influence of the environmental parameters on the CCA biplot are indicated by arrows, which length of arrows indicate their importance (Liu et al., 2009). Tange Dalab and Chogha Sabz had the longest arrow, which indicate they had the most importance in influencing the bacterial community. Disease index was also significantly linked to bacterial taxa variance in CCA. The first axis explained 27.43% of variation and was dominated by Chogha Sabz, Eyvan, Gale Jar and disease index. The second axis explained 16.29% of variation and was dominated by Malek Shahi, Chavar, Tange Dalab, Saleh Abad and stem, root, and rhizosphere sample types. CCA1 was predominately influenced of the variance in bacterial taxa distribution by disease index about 14.95% and Tange Dalab around 11.44%. In addition, In the CCA, the most species were plotted in the centre of the axes. This showed the weak association between those and environmental factors. The ordination diagram showed that the abundance of *Brenneria goodwinii* and *Pantoea agglomerans* are strongly associated with Tange Dalab site. CCA analysis also revealed that DiCh (*Dickeya chrysanthemi*) and SeMa (*Serratia marecescence*) were significantly correlated with Chogha Sabz and leaf sample type (r=0.5 and r=0.42, respectively). PsGr (*Pseudomonas granadensis*) (r=0.45) were significantly correlated with Saleh Abad and rhizosphere sample type (P=0.04). BaPu (*Bacillus pumilus*) (r=0.45) and StMa (*Stenotrophomonas maltophilia*) (r=0.47) were significantly correlated with disease index (P=0.04).

4. DISCUSSION

Causative agents of oak decline in Iran have not been fully determined. The possibility that a bacterial pathogen contributed to Persian oak decline had not been considered a few studies have been performed (Ahmadi et al., 2019; Moradi-Amirabad et al., 2019), although there have been previous reports of bacterial wilt of oak in other countries (Barnard et al., 1998; Scortichini et al., 1993; Brady et al., 2010; Brady et al., 2012; Denman et al., 2012). We explored the cultivable bacteriome of the diseased tissues in oak decline symptomatic trees vs healthy trees, in order to determine similarities and differences in the composition of bacterial communities at the site level, as well as at existing at different stages of disease (healthy vs diseased) and at the sample type levels and also in order to better characterize the distribution patterns of microbes that are pathogen and microbes that could be further exploited as biological control agents against oak diseases. Comparison of microbiomes of healthy vs diseased tissues is one way of filtering potential

candidates. The present study clearly demonstrated highly significant similarities in species composition of symptomatic tissues compared with those in healthy tree tissues irrespective of site. We deliberately chose a culture dependant approach, instead of cultivation-independent metagenomics approaches, because we needed living microorganisms to evaluate their potential to control oak pathogens. The colonization of endophytes bacteria may be influenced by disease index and sampling site (Figure 1). The population densities of culturable bacteria (colonization frequency) in this study obtained in DI=5 (last stage disease) of bulk sample (Figure 1) were similar to the population density of colonies bacterial obtained from symptomatic tissue pieces from diseased trees by Denman et al (2016). But the most diversity of identified bacterial isolates indicated in DI=3 (Figure 4). The high diversity of bacteria in the DI=3 could be because that the trees are metabolizing at this stage of the disease and bacterial species associated with trees including both pathogenic and endophytic bacteria (Frank et al., 2017; Ou et al, 2019; Berg et al., 2019) but reduction of bacteria diversity in DI=5 (Figure 4) could be because the tree is dying out in this sage of disease and often the trees do not have endophytic bacteria and it's likely that bacteria associated with the trees are pathogenic as shown in the results bacterial families in DI=5 are often pathogenic previous work.*Enterobacteriaceae*and*Xanthomonadaceae*family, are known for their degrading capacity of plant tissues (Kandel et al., 2017; IuA et al., 2005; Mhedbi-Hajri et la., 2011; Mitter et al., 2017; Hardoim et al., 2015). Overall, great species richness was observed (Table 1) with few dominant species and many rare taxa, following the community structures in many ecosystems (Magurran and Henderson, 2003). The significance of rare taxa remains unclear, we do not even know if they are active or simply awaiting favorable environmental conditions to become active. The 31 bacteria endophyte isolated in this survey (Table 2), largely corresponds to the numbers obtained in similar studies of oak, ironwood, citrus and apple (Denman et al., 2016; Ayin et al., 2015; Trivedi et al., 2011; Yashiro et al, 2011) indicating relative consistency in endophyte isolation efficiency across studies. The most important oak pathogens oak known from the literatures were isolated and they were among the most abundant species *Brreneria goodwini* ,*Dickeya chrysanthemi* and *Serratia marecescence* ,*Bacillus pumilus* , *Stenotrophomonas maltophilia* (Figure 1). The pathogenicity of these bacterial species on Mediterranean and Persian oak species has therefore been proven (Ahmadi, et al., 2019; Moradi-Amirabad, et al., 2019; Poza-Carrion et al., 2008; Brady et al. 2014a, b). This three species (*Brreneria goodwini* , *Dickeya chrysanthemi* and *Serratia marecescence*) in acute oak decline tissue has been reported previously (Brown, 2013; Denman et al., 2012; Denman et al., 2014; Sapp et al., 2016; Poza-Carrion et al., 2008). To date, it is known that *Dickeya chrysanthemi* has been isolated from potato with twig and stem cankers (as *Dickeya solani*) (Wolf et al., 2014) and *Populus* x *euramericana*with bleeding cankers from the bark (Li et al., 2014), Pea and*Phalaenopsis* orchid as major soft rot pathogens (Ališ et al., 2017; Grenie et al., 2006). Furthermore, the significant association of certain bacterial species with diseased tissues implies that necrosis is not caused by random opportunistic organisms. Remarkably, an important diversity of non-oak pathogenic bacteria was also isolated, of which some tree species (pears, peaches, mangoes, ficus lacor, pine tree, maize and sorghum) have not yet been reported as oak endophytes, such as*Pantoea agglomerans* and *Pseudomonas granadensi* (Li et al., 2009; Saleh et al., 1997; Galal et al., 2006; Hakim et al., 2015; Kovaleva et al., 2015; Proença et al., 2017; He et al., 2016; Arbuzova et al., 2014; Cheng et al., 2013; Coutinho & Venter et al., 2009; Cruz et al., 2007; Morales-Valenzuela, et al., 2007). Since these species was observed in disease indexs 3 and 5 it can be assumed that this species is also pathogenic on oak. Among 148 isolated 38 strains of*Bacillus* (endospore-forming bacteria belonging to the genera*Bacillus* , *Paenibacillus* and *Lysinibacillus*) isolated from different parts of the oak trees (Table 1). They exist in all sites but there were more in the Chavar and Arghavan areas (Figure 3). Notable point the most *Bacillus* species were in low disease index, that's mean in primary disease (index 1 and index 2) situations there were usually dominance of the *Bacillus* species but with low frequency for each species. Except *Bacillus megaterium* and*Bacillus pumilus* that were seen in disease index 5.*Bacillus pumilus* as a pathogen proved in some tree species (oak, pears, peaches, mangoes, Ficus lacor, pine tree) (Li et al., 2009; Saleh et al., 1997; Galal et al., 2006; Hakim et al., 2015; Kovaleva et al., 2015) but about *Bacillus megaterium* no report as a pathogen and often found in rhizospheres and phylloplanes due to its ability to survive and thrive under different nutrient conditions. As these environments tend to be the initial areas screened for biocontrol agents, *B. megaterium* has previously been tested, and has shown the potential to inhibit or suppress a range of plant diseases, occurring on both

the roots and aerial parts of the plant (Islam and Nandi, 1985; Liu & Sinclair, 1992; Pengnoo et al., 2000; Wiwattanapatapee et al., 2004). *Bacillus* genus is abundant in various ecological niches which include soil, water and air (Longan N.A., Halket, 2011; Zhang et al., 2009). Generally, *Bacillus*species used in bio-control and utilized as PGPR (Rais et al., 2017). Probably because of the presence of *Bacillus* species in this sites that disease symptoms have not been recorded on oak. It is of interest to note that a majority of the isolates recovered in primary stage of disease (index 1 and index 2) situations were gram positive bacteria and it has useful role for plant. The described properties of bacteria isolated from healthy stand indicate that the in these samples are dominated by plant-associated and ubiquitously occurring bacteria. Two species, *Clostridium* Sp and *Acinentobactercalcoacelticus* are considered as plant growth promoting rhizobacteria (PGPR), which might be able to control the growth of potentially harmful microorganisms (Mowlick et al., 2012; Park et al., 2005; Rokhbakhsh-Zamin et al., 2011; Sachdev et la., 2010; yang et al., 2001). *Campylobacter jejuni* were isolated from different disease index (DI=1, 3 and 4), *Campylobacter* are gram-negative spiral shaped bacteria known for causing diseases in humans and animals and plant (CDC 2014). The genus *campylobacter* comprises opportunistic pathogens that are frequently isolated from fruits and vegetables, which often are consumed raw (Brandl et al., 2004), so it is interesting to find this pathogen associated with symptomatic and asymptomatic oak trees. *Achromobacter xylosoxidans* formerly known as*Alcaligenes xylosoxidans* is an environmental non-lactose fermenting aerobic motile Gram-negative rod characterized in 1971(Yabuuchi et al., 1974; De Baets et al., 2007). It is an environmental opportunistic human pathogen (Gomila et al., 2014; Jakobsen et al., 2013). On the contrary in many studies have been mentioned to the role as biocontrol (Moretti et al.,2008) and PGPR (Jha & Kumar, 2009; Ma et al.,2009; Bertrand et al., 1999; Abdel-Rahman et al., 2014) and phytoremediation in plant (Ho et al., 2013, 2012).

Results of ordination analysis showed that endophytic bacterial communities were significantly different at a landscape scale (i.e. site differences), but not among sample types (Figure 5, Table 2), result show that the bacteria species composition of Tange Dalab and Gale Jar site is highly specific and Tange Dalab site had the most similar bacteria species composition to Chogha Sabz site (Fig 5 a) bacteria associated with the trees in this sites are often pathogenic base on literature review for example*Enterobacteriaceae*and*Xanthomonadaceae*family and in field observations we also observed that the Tange Dalab and Chogha Sabz was the focus of the contamination. PERMANOVA analysis showed no significant differences between different sample (Table 2) and in Figure 5 b observed points by sample type are mixed on the plot. This suggests no difference in species composition between the different sample type (fig 5b) nevertheless among different sample type the most diversity of identified bacterial isolates indicated in root and stem (Table 1). Some rhizoplane-colonizing bacteria can penetrate plant roots, and some strains may move to stem and leaves, with a lower bacterial density in comparison to root-colonizing populations (Compant et al. 2010). Sturz et al. (1997) also showed that 87% of the endophytic bacteria in the lower foliage of red clover were isolated from inside of roots and root nodules. The lower endophytic bacterial diversity in the leaves and stems could be related to the relatively low diversity of bacteria in the phyllosphere, where they are exposed to stress factors such as UV radiation, desiccation, reactive oxygen and lack of nutrients (Lindow & Brandl 2003). A logical hypothesis is that the life span (i.e. annual or perennial) of the plant species may also influence the diversity and composition of bacterial endophytes in different tissues especially those in leaves, given that most endophytes are derived from soil and migrate from the root to other tissues. Higher endophytic bacterial diversity in roots, and differentiated bacterial communities among leaf, stem and root samples, are clearly supported by our study of a perennial plant species, *Quercus brantii* , which loses its leaves seasonally in temperate climates.

Also Results show that there are highly significant similarities in bacterial species composition of the cultivatable lesion microbiome of diseased trees vs healthy trees (disese index) irrespective of site (Table 2 and Figure 5c) that this result have been also observed in several other studies (Denman et al., 2016; Ayin et al., 2015). The overall health status of the trees (DI=1, 2) had only a very small effect on the general oak microbiome, which indicates that there was no common shift towards a disease indicating community across the sites studied. This implies, that the whole bacterial community does not shift drastically towards a disease indicator microbiome independent of site. Instead, rather small global effects are observed by symptomatic

tissue (DI= 3,4 and 5), presumably masked by location specific signatures. This is supported by the finding, that specific sites did display distinct shifts in oak associated communities for symptomatic tissues, especially in advanced stages when signatures from other sites were removed. This finding implies that the observed shifts are dependent on geographical conditions highlighting that spatial structuring occurs regardless of the studied Acute Oak Decline syndrome being apparent (Denman et al., 2014).

To investigate relationships between bacterial community composition and environmental variables, different bacterial taxa were analysed using CCA (Fig. 6). The sampling site seemed to affect bacterial communities. Results showed *Dickeya chrysanthemi* and *Serratia marecescence* correlated with Chogha Sabz and *Pseudomonas granadensis* correlated with Saleh Abad. Geographical distance appeared to shape the oak microbiome implicating strong spatial structuring and adaptation to the local environment which was also shown previously on Tamarix and oak trees (Finkel et al., 2011; Denman et al., 2016). Suggesting that sampling zone is more important than sample type in structuring the endophytic bacterial community in *Q.brantii* . as expected given the extreme difference of the habitats where they are living, in terms of their degree of exposure (to air, sun, wind, rain, and related moisture and aeration conditions) and availability of nutrients (Andreote et al., 2014). In other hand, some soil properties such as pH in each site are expected to control microbial community composition (Drenovsky et al., 2004). Fierer & Jackson (2006) demonstrated that pH influenced the overall diversity and composition of microbial communities in a range of terrestrial and aquatic environments. Salinity could also affect bacterial composition and diversity across a variety of habitats (Wakelin et al., 2012). Therefore can be concluded that that sampling site is more important in structuring the endophytic bacterial community in *Q.brantii* . as that indicated in Figure 5a and 6.

CONCLUSION

Our study highlights the potential for survey of bacteria endophtyes in Persian oak to reveal of taxa diversity. The ecological significance and potential for use in biological control of many of them are largely unknown. Further research is required to identify the functional and ecological significance of specific endophytic bacteria within the plant under different conditions or at different sites. Our results indicate that the presence of bacteria endophytes in oak can be affected by both site and tissue. For the first time, insights into specific elements of the bacterial microbiomes of both healthy and diseased native oak trees in Iran have been and a culture collection amassed for further study. This study has clearly shown that there is a shift in the cultivable bacterial microbiome from healthy to diseased trees and that the cultivatable microbiome of diseased tissues was not dominated by a single taxon but by three bacterial species. This implies that lesions that characterize declined oak are not caused by a single organism but are likely to have a polymicrobial cause. In light of this, it would be interesting to find out whether similar statistical associations are evident in an even bigger sample, not only from the Iran but from continental Europe as well, where similar disease symptoms have been recorded on oak. Testing is now required to understand the ecological role that these organisms play in oak health and their interactions with each other as well as with insects and the host.

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Data Accessibility

All microbial isolates have been deposited at in the Microbial of Agriculture Biotechnology

Research Institute of Iran culture collections (ABRII CC). Sequences have been deposited in the GenBank database under accession numbers MN149347, MN148622, MN121125, MN120776, MN120784, MN120788, MN120782, MN120783, MN083314, MN083312, MN083309, MN083311, MN083304, MN068411, MN068417, MN068416, MN068414, MN068409, MN068412, MN068408, MN068410, MN068406, MN055978, MN055970, MN055968, MN416308, MN416316, MN416320, MN416322, MN416323, MN416325, MN416399, MN416400, MN416763, MN416949, MN416950, MN416953, MN417054, MN 417506, MN417756, MN418140.

Author Contributions

Elahe Ahmadi: Carried out the experiments, analyzed the data and involved in drafting the manuscript.

Mojegan Kowsari: Supervised the project , designed the experiments and involved in drafting the manuscript.

Davoud Azadfar: Revised the manuscript.

Gholamreza Salehi Jouzani: Participated in sampling and revised the manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

Species (Family)	Codes isolate***	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sites**	Number of isolates in different sites**	Num of iso- lates in dif- fer- ent sites
		1	2	3	4	5	Le	Ro	St	Rh	Bu	G	A	E
Achromobacter Xylosoxidans (Alcaligenaceae)	AdXy	0	5	6	0	0	0	3	0	5	3	6	5	0
Acinetobacter Calcoaceticus (Moraxellaceae)	AbCa	8	0	0	0	0	2	5	0	1	0	0	5	2

Species (Family)	Codes for isolate***	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in different samples*	Number of isolates in different samples*	Number of isolates in different samples*	Number of isolates in different samples*	Number of isolates in different sample*	Number of isolates in different sites**	Number of isolates in different sites**	Num of isolates in different sites
Bacillus Cereus (Bacillaceae)	BaCe	1	3	0	0	0	2	0	2	0	0	2	1	0
Bacillus Endophyticus (Bacillaceae)	BaEn	0	1	0	0	0	0	1	0	0	0	0	1	0
Bacillus Megaterium (Bacillaceae)	BaMe	0	0	0	0	1	0	1	0	0	0	0	1	0
Bacillus Methylotrophicus (Bacillaceae)	BaMet	1	1	0	0	0	0	0	0	0	2	1	1	0
Bacillus Mojavensis (Bacillaceae)	BaMo	0	0	3	0	0	2	0	1	0	0	1	1	0
Bacillus Mycoides (Bacillaceae)	BaMy	0	1	0	0	0	0	0	1	0	0	0	1	0
Bacillus Pumilus (Bacillaceae)	BaPu	0	0	0	2	3	1	2	2	0	0	0	0	0
Bacillus Safensis (Bacillaceae)	BaSa	0	2	0	0	0	1	1	0	0	0	1	0	0
Bacillus Simplex (Bacillaceae)	BaSi	1	2	0	0	0	0	0	3	0	0	1	0	1
Bacillus Subtilis (Bacillaceae)	BaSu	2	2	0	0	0	1	1	2	0	0	2	0	1

Species (Family)	Codes	Isolate***	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in different samples*	Number of isolates in different samples*	Number of isolates in different samples*	Number of isolates in different samples*	Number of isolates in different sample*	Number of isolates in different sites**	Number of isolates in different sites**	Num of isolates in different sites
Bacillus Thuringiensis (Bacillaceae)	BaTh		0	0	1	0	0	1	0	0	0	0	0	0	1
Bacillus Toyonensis (Bacillaceae)	BaTo		0	1	0	0	0	0	0	1	0	0	0	0	0
Bacillus Vallismortis (Bacillaceae)	BaVa		1	0	1	0	0	1	0	1	0	0	0	1	0
Bacillus Velezensis (Bacillaceae)	BaVe		1	1	0	0	0	1	0	1	0	0	0	1	0
Brenneria goodwinii (Pectobacteriaceae)	BrGo		0	0	0	6	0	2	0	4	0	0	0	0	4
Brevibacillus Invocate (Paenibacillaceae)	BrIn		0	1	2	0	0	0	0	0	2	1	0	0	2
Brevibacterium Frigoritolerans (Brevibacteriaceae)	BrFr		0	0	1	0	0	0	0	0	1	0	0	0	0

Species (Family)	Codes	Isolate***	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sites**	Number of isolates in different sites**	Num of isolates in different sites
Brevibacterium Halotolerans (Brevibacteriaceae)	BrHa		0	0	0	1	0	0	0	0	1	0	0	0	0
Campylobacter Jejuni (Campylobacteraceae)	CaJe		3	0	4	5	0	0	4	3	5	0	7	0	3
Clostridium Sp (Clostridiaceae)	ClSp		2	0	3	3	0	2	1	0	0	5	5	2	0
Dickeya Chrysanthemi (Enterobacteriaceae)	DiCh		0	1	0	3	5	1	2	4	0	2	0	0	3
Lysinibacillus Fusiformis (Bacillaceae)	LyFu		1	1	0	0	0	1	1	0	0	0	0	0	0
Lysinibacillus Xylanilyticus (Bacillaceae)	LyXy		0	0	2	0	0	1	1	0	0	0	0	2	0
Microbacterium Arborescens (Microbacteriaceae)	MiAr		0	3	2	0	0	1	0	1	1	2	2	0	0
Paenibacillus Apiarius (Paenibacillaceae)	PaAp		2	1	0	0	0	0	1	2	0	0	0	0	0

Species (Family)	Codes for isolate***	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in disease indexs	Number of isolates in different samples*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sample*	Number of isolates in different sites**	Number of isolates in different sites**	Num of iso- lates in dif- fer- ent sites
Pantoea Agglomerans (Enterobacteriaceae)	PaAg	0	0	5	0	9	0	4	6	0	4	0	0	0
Pseudomonas Granadensis (Pseudomonadaceae)	PsGr	0	0	3	3	1	0	0	0	3	4	0	0	0
Serratia Marcescence (Enterobacteriaceae)	SeMa	0	0	2	3	4	2	0	0	3	4	0	0	0
Stenotrophomonas Maltophilia (**Xanthomonadaceae**)	StMa	0	0	3	5	7	9	6	0	0	0	0	0	0

TABLE 1 Species of bacteria isolated from different samples of the five disease index in eight site

*Le: Leaf; Ro: Root; St: Stem; Rh: Rhizosphere, Bu: Bulk

**G: Gale jar; A: Arghavan; E: Eyvan; S: Sale abad; C: Chavar; O: Chogha sabz; T: Tange dalab; M: Malek shahi.

***Species codes are first three letters of genus + first three letters of specific epithet

TABLE 2 Statistical testing of bacterial community composition in response to different environmental variables.

Variation/index	Bacterial community composition	Bacterial community composition	Bacterial communit
	Sum of Sqs	R^2	F-value
Site	0.0173	0.269	4.4769
Disease index	0.0034	0.053	5.103
Sample type	0.0035	0.055	1.373
Site× Disease index	0.0064	0.1007	1.675
Sample type × Disease index	0.0029	0.0459	1.090
Sample type × Site	0.0180	0.280	1.246

Mean significant in different probability level: Signif. codes: 0 '***'; 0.001 '**'; 0.01 '*'; ns not significant.

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