Plant Soil
https://doi.org/10.1007/s11104-024-07198-1

RESEARCH ARTICLE

In vitro domestication of halophyte microbiota for future SynCom application

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Received: 1 October 2024 / Accepted: 23 December 2024

Abstract

Background and aims Microbiome-mediated strategies for future stressed-agriculture entail exploration of repertoires of halophyte microbiota. Culturomics strategies are advanced to improve culturability and extend diversity of microbiota of *Salicornia europaea* L.

Methods The plant broth-based-seawater-culture medium (PBSW) was advanced for in vitro domestication of microbiota of endo-rhizosphere/endo-phyllosphere of *S. europaea*. Populations (Colony Forming Units, CFUs) and biomass production (Optical Density, OD) were monitored throughout successive steps of in vitro cultivation/domestication in liquid batch cultures. Culture-dependent methods were applied to cultivate and identify (16S rRNA gene sequencing) representative isolates; and culture-independent analyses (DGGE/qPCR) for community composition.

Results PBSW supported higher CFUs counts; and related to 16S rRNA gene copy numbers (qPCR), increased (> 40 fold) culturability compared to NaCl-salted-standard culture medium. Successive in vitro domestication/batch cultures boosted bacterial growth, diminished differences among tested culture media and shortened doubling times (DT). PCR-DGGE showed divergence in culturable community composition primarily attributed

Responsible Editor: Nabil Abrahi Hegazi.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s11104-024-07198-1.

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Published online: 15 February 2025

to culture media. 16S rRNA gene sequencing of representative isolates indicated: a) greater diversity in endo-phyllosphere than endo-rhizosphere; b) abundant phyla were Pseudomonadota/Bacillota /Actinomycetota; c) dominance of *Halomonas* among 15 genera identified; d) *Gracilibacillus, Metabacillus, Mixta, Salinicoccus, Zhihengliuella, Marinobacter, Marinimicrobium* and *Planomicrobium* were first reported/cultivated for *S. europaea*. In vitro domestication resulted in dominance of genera of Pseudomonadota/Bacillota for endo-phyllosphere and *Halomona*s sp. of Pseudomonadota for endo-rhizosphere.

Conclusion PBSW created in situ *similis* milieu for cultivation of halophyte bacteria, and enabled in vitro domestication for propagating microbiota, instead of laborious construction of consortia of single isolates, for future SynCom applications.

Keywords Halophyte microbiota · *Salicornia europaea* · Microbiota in vitro domestication · Culturomics of halophyte microbiota · Mariout lake-Alexandria-Egypt halophytes · Biomass production of halophyte microbiota

Introduction

It is well established that soil salinity represents an environmental constraint that challenges agricultural productivity worldwide. Singh (2022) reported that more than 800 million hectares of the world lands are salt-affected; and due to global climate changes and the elevation of sea level will certainly threaten agricultural productivity. He discussed two main reasons for soil salinization: a) human-made, e.g. irrigation of crops with low-quality waters and prolonged dry spells together with heavy chemical fertilizers application, and b) natural salinization due to the natural soil parent materials and physical/chemical weathering of minerals as well as seawater intrusion. Those stresses markedly changed the agricultural lands towards reduced productivity, resulting in potential loss of production of *ca.* 20% all over the world land (Ji et al. 2022).

A number of plants, designated as halophytes, are well-adapted to grow and reproduce in salt-stressed soils and sediments under salinity levels more than 200 mM NaCl (Yuan et al. 2016). Mechanisms for salinity tolerance of halophytes are reported to include: reduced ion transport and uptake, maintenance of redox and energetic status, ion compartmentalization, osmotic adjustment and succulence in addition to salt inclusion/excretion (Lokhande et al. 2013).

Salicornia europaea L. (Glasswort) is an annual halophyte of the Amaranthaceae family, and considered among the highly salt- tolerant halophyte species that can withstand salt concentrations up to 1000 mM (Cárdenas-Pérez et al. 2022; Lv et al. 2012). This particular halophytic plant is considered an appropriate model plant for investigating the response to salt stress besides the efficiency of desalination (Muscolo et al. 2014). It is distributed worldwide and in north/west Europe exists the native range of this species. Primarily, the plant is common in the temperate biome but extends to Egypt and Middle East and North Africa (MENA)region (Davy et al. 2001; POWO 2024; Santos et al. 2016). This species possesses multifunctional and multiple therapeutic applications (Gu et al. 2023). In addition, the plant is gaining economic value as gourmet food products, fodder for animals, oil seeds and raw materials possibly used for the extraction of dietary fibres, besides high added-value by-products (Cárdenas-Pérez et al. 2021; Rhee et al. 2009). Based on the fact that living plants are densely populated with microbial counterparts, it is expected that salinity tolerance mechanisms of halophytes are related /connected with halotolerant microbiota. Unfortunately, not enough attention has been given to *S. europaea* microbiome in relation to salinity stress, particularly in salt-affected environments in Egypt and MENA region.

Culture-independent microbiota analyses proved that less than 1% of the microbial species in various environments, e.g. seawater and marine sediments, have been cultivated (Bodor et al. 2020; Pace 1997). Here, the need arises for more advanced procedures and techniques to monitor the diversity of endophytic microorganisms of all plants, including halophytes. During the last few years, culture-independent approaches have been introduced to trace and assess the community composition of endophytes nesting the internal tissues of different plant species (Demko et al. 2021; Hinsu et al. 2021; Sarhan et al. 2019; Su et al. 2012; Thompson et al. 2017). These techniques

indicated conspicuous differences in the composition of bacterial communities associated with the different plant compartments. Higher diversity was reported in the ecto-rhizosphere than endo-rhizosphere (Gao et al. 2022) and at internal root tissues compared to inside shoots (Furtado et al. 2019). More interestingly, the fine structure of microbiomes is largely imprinted by soil salinity level (Szymańska et al. 2018), and salinity possibly imposes a degree of intraspecific variability in the structure and composition of the root-associated microbiota (Hrynkiewicz et al. 2019). Such microbiota is considered a significant repertoire of diverse communities dominated by representatives of the major phyla: Pseudomonadota, Bacillota, Bacteroidota and Actinomycetota (Dragojević et al. 2023; Gao et al. 2022; Hrynkiewicz et al. 2019; Mapelli et al. 2013; Szymańska et al. 2016). In particular, Proteobacteria are reported of greater abundance among such halophyte's microbiota designating their importance to the adaptation of halophytes to existing saline habitats (Ahmed et al. 2018; Fidalgo et al. 2016; Furtado et al. 2019)**.** Through multiple functions, e.g. plant growth promoting substances (PGP), they improve plant nutrition and health under extreme salt-stressed conditions (Dragojević et al. 2023). Actually, this particular information not only expand the awareness on the role played by root-associated bacterial species to the adaptation of *Salicornia* spp. to salt stresses but may, as well, support the tailored design of beneficial microbial consortia to be introduced/adapted with host plants grown in marginal environments.

The synthetic community (SynCom) approach represents an emerging research field that encompasses a construction of synthetic microbial communities using the acquired knowledge from microbiome analyses, based on metagenomics and bioinformatics approaches (McCarty and Ledesma-Amaro 2019). SynCom could be constructed via co-culturing multiple microbial taxa under well-controlled and defined conditions to mimic the structure and function of a given microbiome community. This is in an attempt to facilitate the community stability by fostering synergistic relationship between the existing members (de Souza et al. 2020). Several studies proved remarkable promotion for plant growth due to SynCom applications. Armanhi et al. (2018) reported significant increases in maize biomass yield due to inoculation with a SynCom constructed from sugarcane-associated microbiota. As well, the technology improved drought tolerance and reduced the yield loss of the cereal plants (Armanhi et al. 2021). In a field trials, the functionally assembled SynCom magnified soybean yield by *ca*. 36% (Wang et al. 2021).

Since the early work of Charles Darwin in 1868 "*The Variation of Animals and Plants under Domestication;* https://www.britannica.com/topic/The-Variation-of-Animals-and-Plants-Under-Domestication *"*, microbial domestication has long been overlooked. In general, domestication is defined as the adaptation over time, principally by selective breeding, from a wild state of life in association with the human benefit. During domestication, certain morpho-physiological changes are occurring that distinguish domesticated taxa from their wild ancestors (Lye and Purugganan 2019). Being different from plants and animals, domestication of microbes occurred on populations of millions of cells, leading to less-controlled domestication (Steensels et al. 2019). Therefore, domestication of microbes encompasses continuous and fierce competition between different variants (species, strains, mutants, etc.) in each domestication round. With the new developments of manipulation of single cells and pure cultures, together with new biotechnological techniques such as genome shuffling or protoplast fusion, opportunities are available to perform more 'traditional' domestication in microbes (Steensels et al. 2019). Although microbial domestication is highly linked to our common lives, a number of questions need to be answered: how, when and why does microbial domestication happens? Therefore, research on microbial domestication is a real challenge.

In the present study, the halophyte community of *Salicornia europaea* inhabiting the salt-stressed environment of north-western part of the Mediterranean coast, Alexandria- Egypt was surveyed. Fresh representative samples were collected to explore the diversity of its associated microbiota, using the natural/compatible host plant broth based –sea water culture medium in comparison to salted-standard chemically synthetic culture medium. Culture-dependent (CFUs) and –independent (qPCR, DGGE) analyses were implemented to analyse the prevailing salt-tolerant and culturable bacterial communities in plant compartments, endo-rhizosphere and endo-phyllosphere. 16S rRNA gene

sequencing of representative isolates was performed for identification and diversity indication. Domestication of in situ endo-rhizosphere/ endo-phyllosphere microbiota was undertaken in batch cultures of tested culture media and along three successive steps. Growth, in terms of OD, doubling times and CFUs counts, as well as physico-chemical measurements were monitored. This is to follow and identify the in vitro-domesticated bacterial communities in comparison to those present in the in situ environment. The major objective is to reproduce the in vitro domesticated taxa that can be further used as SynCom for future introduction to economic crops grown under salinity stress.

Materials and Methods

Hypothesis and experimental design

The main objective of this study was to investigate the possibility of in vitro domestication and increasing culturability of halophyte microbiota associated to *Salicornia europaea.* For this purpose, plant microbiota of endo-rhizosphere and endo-phyllosphere were grown in successive liquid batch cultures prepared from compatible plant broth-based sea water culture medium compared to synthetic culture medium salted with NaCl. The developed culturable communities were compared in terms of CFUs, biomass production (OD), and composition (DGGE analysis). Representative isolates were secured and identified through 16S rRNA gene sequencing. They were further tested for culture media preference and elevated salt tolerance (Fig. 1).

The sampling site and tested plants

The salt-stressed wild-plant environment of Mariout Lake was surveyed for *Salicornia europaea.* The area is located at north-western part of the Mediterranean coast, Borg El-Arab regions, Alexandria governorate—Egypt (N 30°59′29.2488" E 29°39′10.9188"). The site is dominated by communities of several obligate halophytic species (Fig. 2), including the common glasswort; *Salicornia europaea* L., that belongs to the family Amaranthaceae. Representative two biological samples representing two locations, each comprised of 2 plants at the fruiting stage (Fig. 2 C) collected in the middle of October, 2022.

Sampling of soil- plant system and nearby sea water

Plant samples were collected by first insertion and separation of the vegetative parts (phyllosphere) into sterilized dark plastic bags. Intact roots with closely adherent soil were gently-uprooted as well and transferred to sterilized plastic bags. Besides, free soil samples nearby the roots were collected. In addition, samples of nearby sea water from Mariout Lake were obtained. Samples were brought to the laboratory and kept in refrigerator at + 4 °C. Within 48 h., representative two samples of soil and water were subjected for physico-chemical analyses (Table S1) according to procedures of Jackson (1973); Richard (1954); and Saleh et al. (2017). Plant samples were subjected to microbiological analyses as indicated below.

Culture media preparation

Standard N-deficient combined carbon sources culture medium (CCM)

This chemically-synthetic culture medium, introduced by Hegazi et al. (1998) satisfies the nutritional requirements for a variety of rhizobacteria where it contains a limited N level with diverse carbon sources that mimic the root *milieu*. It consists of (g L^{-1}): glucose, 2.0; malic acid, 2.0; mannitol, 2.0; sucrose,1.0; K_2HPO_4, 0.4; KH_2PO_4, 0.6; $MgSO_4$, 0.2; NaCl, 0.1; $MnSO_4$, 0.01; yeast extract, 0.2; fermentol (a local product of corn-steep liquor),0.2; KOH, 1.5; $CaCl_2$, 0.02; $FeCl_3$, 0.015; Na_2MoO_4, 0.002. In addition, $CuSO_4$, 0.08 mg; $ZnSO_4$, 0.25 mg; sodium lactate, 0.6 mL (50% v/v) were added per litter. The culture medium was adjusted to pH 7.0, then diluted (1:10, w/v) and supplemented with sodium chloride as 30 g L^{-1} (EC 56 dSm^{-1}, identified as 1/10 CCM 30).

Plant broth-based-sea water culture medium (PBSW)

The procedure of preparation of plant broth described by Elsawey et al. (2020) was adopted. Coarse-chopped areal parts of *Salicornia europaea* plant were thoroughly washed with sea water, then

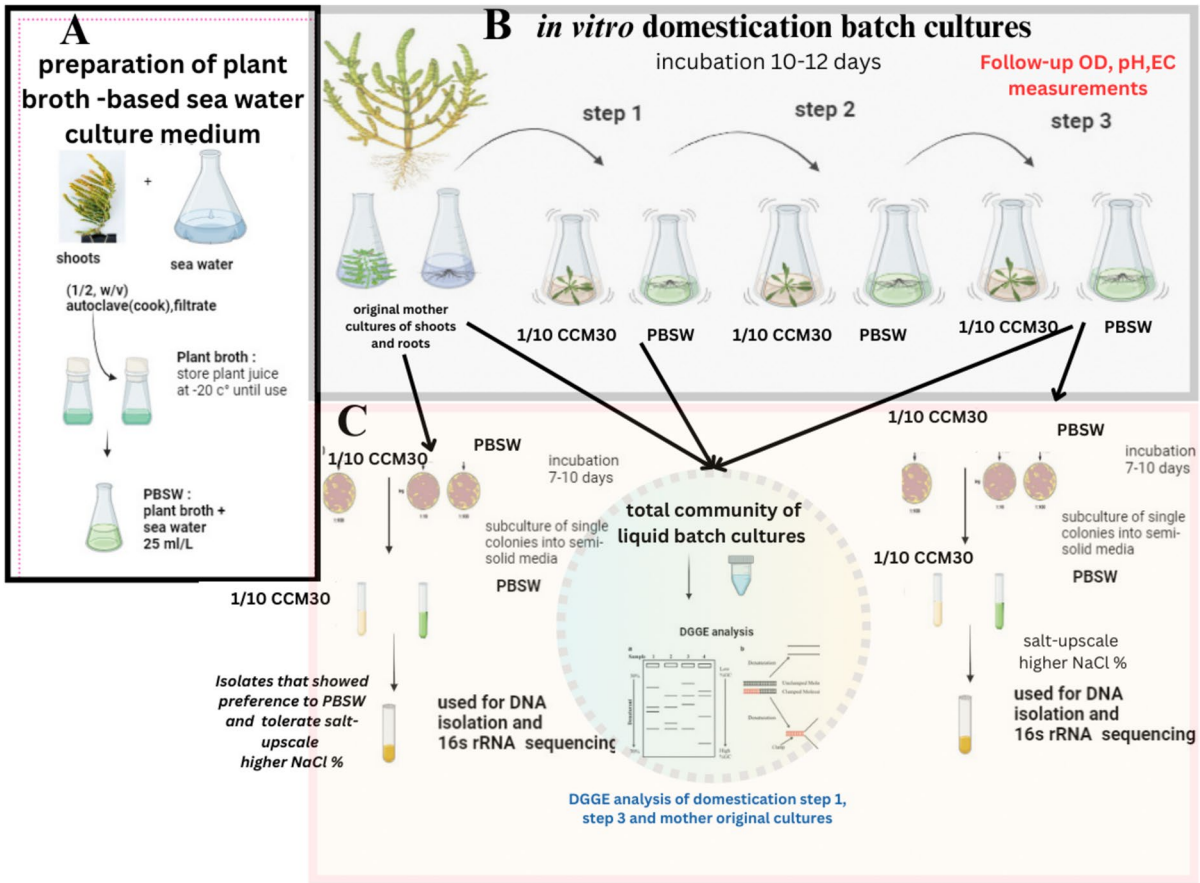


Fig. 1 Schematic illustration of the various steps of in vitro domestication: **A** preparation of the plant broth-based sea water culture medium (PBSW); **B** successive in vitro batch culturing; **C** various analysis and measurements that included CFUs counting, isolation of representative isolates for 16S rRNA sequencing, and DGGE profiling

transferred to Erlenmeyer flasks containing sea water (1/2, w/v) of EC 68.6 dSm^{-1} and pH 8.20. After heat-extraction in autoclave at 121 °C for 20 min., the mixture was pressed and filtered twice through cotton cloth and Whatman No. 1 filter paper. The plant broth stock characterized by pH 6.86 and EC 59.30 dSm^{-1} was stored at −20 °C. The plant broth-based-sea water culture medium was prepared by the addition of plant broth to sea water (25 ml L^{-1}) and autoclaved at 121 °C for 20 min. (Fig. 1). The pH of the resultant culture medium was 7.67, and EC was 68 dSm^{-1}.

Culture-dependent analyses of halophyte microbiota associated to *Salicornia europaea*

The in situ halotolerant culturable microbiota population associated to *S. europaea* was monitored using both diluted synthetic CCM culture medium (1/10 CCM30) and plant broth-based sea water culture medium (PBSW). The endophyte pool was examined for two surface- sterilized plant compartments; endo-phyllosphere and endo-rhizosphere. Here, representative shoot and root samples were surface- sterilized by immersion in 95% ethanol for 1 min. followed by 3% sodium hypochlorite for 30 min., then washed several times with sterilized sea water, 5 min. for each wash, prior to crushing in Waring blender with sufficient amount of sea water (Youssef et al. 2004; Saleh et al. 2017). Original suspensions of plant materials (10 g in 180 ml sterilized sea water, referred to as the mother culture) were prepared and thoroughly mixed by shaking (120 rpm) for 30 min. Serial decimal dilutions using sea water (10^{-1} to 10^{-5})

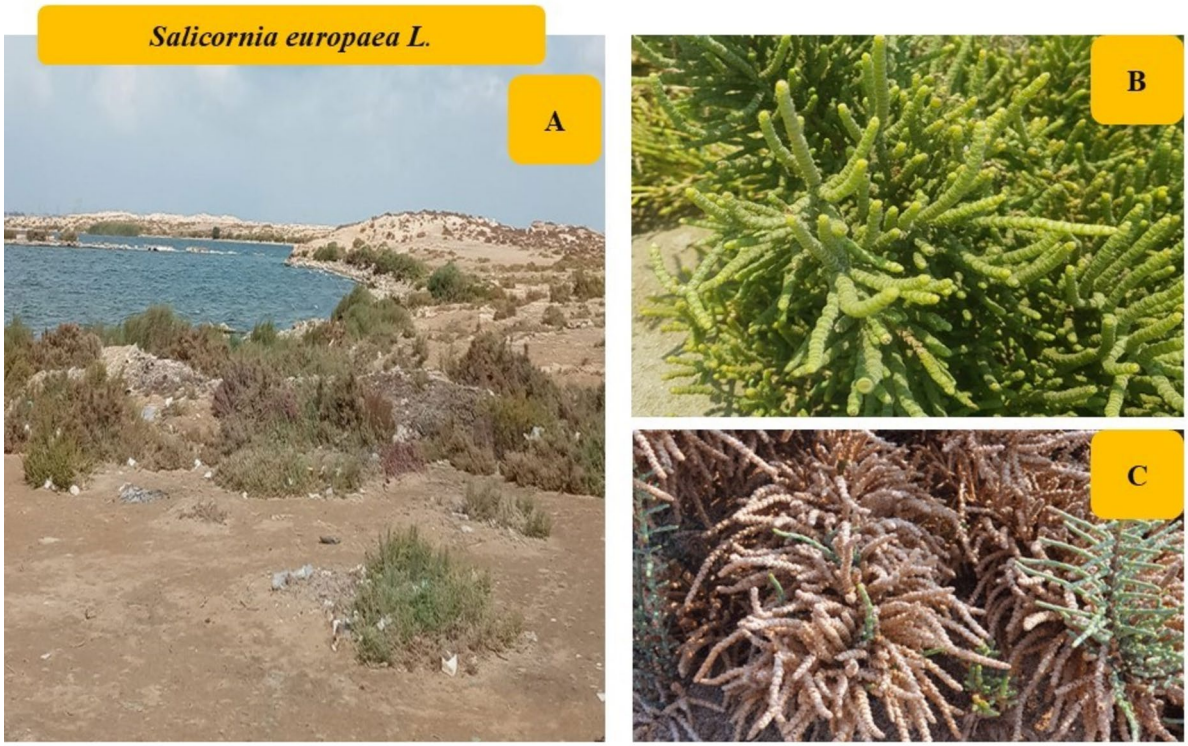


Fig. 2 The studied field site at Mariout Lake, north-western coast, Alexandria-Egypt: overall view (**A**); *Salicornia europaea* plants, vegetative part (**B**) and fruiting branches (**C**)

were prepared. Aliquots (200 µl) of all dilutions were surface-inoculated on agar plates of both culture media (PBSW, 1/10 CCM30). Incubation took place at 25 °C for a period extended to 12 days, and developed CFUs were enumerated. Suspended plant materials in mother cultures were oven-dried at 70 °C and weighed.

In vitro-domestication of *Salicornia europaea* core microbiota in liquid batch cultures

The endo-phyllosphere and endo-rhizosphere core microbiota of *S. europaea* were in vitro-domesticated through three successive enrichment steps in liquid plant broth-based-sea water culture medium (25–50 ml plant broth L^{-1} sea water) in comparison with diluted standard CCM30 (1/10 CCM 30) amended with 3% NaCl (w/v) identified as CCM30. In the first step, 20 ml of initial suspensions (original mother culture) of either endo-phyllosphere or endo-rhizosphere were transferred to flasks containing 180 ml of both liquid culture media 1/10 CCM 30 and PBSW (25 plant broth ml L^{-1} sea water) with incubation at 25 °C for 10- 12 days. For the second step, 180 ml of both test liquid culture media were inoculated with 5 ml of the resulting batch culture media of the first enrichment step, followed by incubation at 25 °C for 10–12 days. Similarly, the third enrichment step was prepared and incubation took place at 25 °C for 10- 12 days. In general, two sets were prepared representing two biological replicates, i.e. two different plants, and two technical replicates were prepared for each of the tested culture media. Incubation was accompanied by intermittent shaking at 120 rpm. At regular intervals of 3, 5, 7, and 12 days, culture media of the three enrichment steps were subjected to optical density (OD_{600}), pH,

and EC measurements; together with calculating the doubling time (Zahari et al. 2022). CFUs counting was additionally carried out for the latest step, step 3, of in vitro-domesticated liquid batch cultures.

Isolation of representative halophytic microbiota associated to *Salicornia europaea* and those prevailing in domesticated batch cultures

Throughout the bacteriological analysis, agar plates representing the various plant compartments, culture media, mother samples and in vitro -domesticated batch cultures were selected. All CFUs developed on each representative agar plate were single colony isolated on corresponding semi-solid culture media (1.85 g agar L^{-1}) to secure a representative and comprehensive pool of pure isolates (Table S2). In general, isolates were further sub-cultured on the same semi-solid culture media with incubation at 25 ℃ for 5–7 days. Visible growth of successfully sub-cultured isolates was macro-/microscopically monitored. Special preference was given to isolates that: a) developed on PBSW agar plates; b) showed preferred growth in semi-solid PBSW compared to 1/10 CCM 30; c) continued better growth on higher levels of salt (10%, 30% NaCl), i.e. salt upscale. A total of 154 isolates representing both plant compartments and various growth conditions were successfully sub-cultured and further subjected to 16S rRNA gene sequencing.

Biomass upscaling

To intensify and upscale biomass production of the in vitro-domesticated core microbiota, we experimented endo-rhizosphere samples growth in presence of higher plant broth concentrations, i.e. more plant nutrients, in the prepared PBSW batch cultures (50 and 200 $mL^{-1}L$ culture media). Various cultivation conditions were examined, including different inocula doses (10 and 20% of the final volume of batch cultures %, v/v), temperatures (25 and 30 °C), and continuous or intermittent shaking (120 rpm).

16S rRNA gene sequencing and phylogenetic affiliation

The total representative 154 isolates mentioned above were subjected to DNA extraction for 16S rRNA gene sequencing. Isolates were grown on PBSW culture medium and incubated at 25 C for 5–7 days. Then bacterial cells were harvested by centrifugation at 9500×g for 15 min., and DNA was extracted from bacterial pellets using the CTAB DNA extraction method (Handiyanti et al. 2018; Joko et al. 2019). The extracted DNA was used as a template to amplify the whole 16S rRNA gene using the primer F27 (AGA GTTTGATCMTGGCTCAG) and R14941514 (CTA CGGYTACCTTGTTACGAC) (Elsayed et al. 2021). The reaction was performed in a total volume of 25 µL with 2 µL template DNA (ca. 2–18 ng mL1), 12.5 µL of QIAGEN TopTaq master mix (Qiagen, Hilden, Germany), 5.5 µL PCR water, and 2.5 µL of 3.3 pmol of both primers, using the Bio-Rad C1000 Thermal Cycler (Bio-Rad, CA, USA). The thermal cycling program was adjusted as follows: 4 min of initial denaturation at 96 ◦C, 30 thermal cycles of 1 min denaturation at 96 C, 1 min annealing at 56 C, and 1.5 min of extension at 72 C; PCR was finished by a final extension step at 72 C for 10 min. QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used to purify the PCR product according to the manufacturers' instructions. 16S rRNA gene sequencing was performed according to Sanger enzymatic sequencing (Eurofins Genomics, Köln, Germany). The resulting 16S rRNA gene sequences were compared with their closest matches in GenBank (www.ncbi.nlm.nih.gov/BLAST/) and EZ-biocloud (https://www.ezbiocloud.net/) databases to determine the taxonomy of the bacterial strains. The phylogenetic trees were constructed via a neighbor-joining method using Clustal Omega (Goujon et al. 2010; Sievers et al. 2011). The 16S rRNA gene sequences identified in this study have been deposited in the NCBI GenBank database under the accession numbers: PP267189-PP267257 (endo-phyllosphere) and PP334640-PP334724 (endo-rhizosphere) (Table S3).

Real-time PCR analysis for quantification of total bacterial counts in representative samples of the endo-phyllosphere

Copy number quantification of 16S rRNA gene was performed (Sarhan et al. 2016) by quantitative real-time PCR using the CFX96 Touch™ Detection System (Bio-Rad Inc., CA) in optical grade 96 well plates. Portions of the original shoot suspensions and domesticated step1 and step 3 of both culture media (1/10 CCM 30, PBSW) were centrifuged at 9500 xg

for 15 min., and then DNA was extracted using the QIAGEN DNeasy® power soil® kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was 1:10 (v/v) diluted and analyzed in triplicates (Saleh et al. 2017). The PCR reaction was performed in a total volume of 10 µL using 5 µL of SsoAdvanced Universal SYBR green Supermix master mix (Bio-Rad, CA, USA) containing 1 µL DNA (ca. 10–100 ng), 0.25 µL of 2.5 pmol both primers of each of the universal forward 519f (CAGCMGCCGCGGTAANWC) and reverse 907r (CCGTCAATTCMTTTRAGTT) primers (Ruppel et al. 2007), and 3.5 µL PCR water. The standard curve was constructed using 478 bp length fragment of purified PCR product of the *Kosakonia radicincitans DSM16656T* 16S rRNA gene in tenfold dilutions with the range of 2.070E + 02–2.070E + 08. The amplification of DNA was done according to the thermal amplification cycling program: 3 min of initial denaturation at 95℃, 35 thermal cycles of denaturation at 95 ℃ for 15 s, annealing at 53 ℃ for 30 s, and extension at 72 ℃ for 42 s; followed by a final extension step at 72 ℃ for 5 min. Finally, a melting curve construction was performed by increasing the temperature from 55 to 95 ℃ with fluorescence detection every 0.5 ℃ to verify the PCR quality. The bacterial cell numbers were obtained indirectly assuming 3.6 is the average number of rRNA operon (Klappenbach et al. 2000; Schippers et al. 2005).

Total community DNA extraction and DGGE analysis

Total community DNA (TC-DNA) was extracted from mother cultures prepared for original surface-sterilized roots/shoots. As well, samples of 20 ml representing in vitro-domesticated batch cultures of steps 1 and 3 of both culture media were included in the analysis. Three replicates were prepared from each sample and centrifuged at 13,000 rpm for 10 min. DNA was extracted from the resulting pellets using the QIAGEN DNeasy® power soil® kit. DNA quality was checked using the BioDrop µLITE spectrophotometer (Biochrom, Holliston, MA, USA). For amplification of 16S rRNA gene and DGGE fingerprinting, the protocols for 16S rRNA gene amplification and nested PCR of the V3-16S rRNA gene were followed by using the 9bfm (GAG TTTGATYHTGGCTCAG) and 1512r (ACGGHTAC CTTGTTACGACTT) primers (Elsawey et al. 2023; Muyzer et al. 1993; Mühling et al. 2008; Sarhan et al. 2016) were used. 16S rRNA gene amplification from DNA extracted was performed with the SensoQuest Thermocycler (SensoQuest, Göttingen, Germany). To obtain the PCR product of the V3 region, 2 µL of the purified 16S rRNA PCR product (10 ng µL − 1) were re-amplified using the 341f-GC (CGCCCGCCG CGCGCGGCGGGCGGGGCGGGGGCACGGGGC CTACGGGAGGC-AGCAG) and 518r (ATTACC GCGGCTGCTGG) primers; the reaction conditions and thermal cycling program were used as described above (Elsawey et al. 2023). DGGE was performed using the VS20WAVE-DGGE (Cleaver Scientific Ltd, Warwickshire, UK). Aliquots of 10 µL of each sample were mixed with 3 µL of a 6X loading dye (glycerin, xylene cyanol, bromophenol blue). Amplicons were electrophoresed using 40% acrylamide gel containing a 30 to70% denaturing gradient of formamide and urea with 1 × TAE buffer. After 5 min of initial migration at 200 V to push the sample into the gel, DGGE was conducted at 60 ˚C for 20 h. at 50 V. The gel was stained for 30 min with the 6X ethidium bromide, photographed, and analyzed for DGGE band profiles with the Micro-DOC System with UV Transilluminator (Cleaver Scientific Ltd, Warwickshire, UK) (Fig. S1).

Data analyses

STATISTICA (Statsoft, Inc. Tusla, USA, Version 10.0 http://www.statsoft.com) was used for the analysis of variance (ANOVA) to examine the significant effects. The phylogenetic trees were annotated using the online tool Interactive Tree of Life (iTOL) (http://itol.embl.de). R version 4.0.2 (https://www.r-project.org/), R-studio (https://www.rstudio.com/), R-project packages (cran.r-project.org), "ggplot2" and "scales" were used for constructing pie charts and stalked columns bars. The DGGE fingerprints were analyzed using GelJ software v.2.0 (Heras et al. 2015).

Results

Culturability of *S. europaea* microbiota as affected by plant compartments and culture media

Microbiota existed in plant compartments of endorhizosphere/ endo-phyllosphere, mother cultures, of *S. europaea* with dense populations (> log 4 – log 7

CFUs g^{-1}). Significant single effects were attributed to a number of factors (Table 1). Prolonged incubation resulted in significant increases in developed CFUs numbers, being highest (log 5.59—5.69 CFUs g^{-1}) at 8–12 days of incubation. As to plant compartments, the endo-rhizosphere hosted higher (log 6.47 CFUs g^{-1}) populations compared to the endo-phyllosphere (log 4.65 CFUs g^{-1}). Of interest is the culture media single effect where the plant broth-based sea water culture medium (PBSW) supported higher culturability, approached > tenfold increases over the standard chemically-synthetic culture medium (1/10 CCM 30). Such effect is extended to ANOVA two-way interactions (Table 1). PBSW favoured the growth of microbiota nested both the endo-rhizosphere and endo-phyllosphere; however, highest increases in CFUs counts were reported for microbiota of endo-phyllosphere compared to the endo-rhizosphere.

The bacterial 16S rRNA gene copy numbers per gram of dry *S. europaea* shoot were determined by qPCR. The mean log of calculated bacterial cell numbers obtained for fourteen replicates was 7.11 ± 0.09, taking into consideration the inherent limitation of the used method and primers of amplification of organelle DNA. The culture-dependent method, in terms of CFU numbers developed on agar plates of the chemically synthetic (1/10 CCM 30) medium represented 0.05% of the qPCR calculated bacterial cell numbers. The plant medium (PBSW) dramatically improved culturability up to 2.19%, representing more than 40 folds of that reported for 1/10 CCM 30 medium (Table 2). The obtained results indicate the suitability of PBSW culture medium to significantly increase the culturability of in situ endo-phyllosphere microbiota. Increasing culturability on PBSW was extended to growth in batch cultures of in vitro domestication/cultivation. With in vitro domestication step 3, PBSW supported better developments and significantly increased counts of CFUs compared to 1/10 CCM 30. Such effect was demonstrated as single effect of culture media as well as interaction with plant compartments (Table 3).

In vitro domestication and biomass production of halophyte microbiota associated to *Salicornia europaea*

Liquid batch cultures prepared from the tested culture media, both 1/10 CCM 30 and PBSW, were prepared for plant samples represented compartments, endo-phyllosphere and endo-rhizosphere. Three successive steps of in vitro domestication were performed (Fig. 1). As to plant compartments, microbiota of endo-phyllosphere were progressively in vitro domesticated yielding statistically higher OD values of 0.260 to 0.505, compared to the endo-rhizosphere (0.198 to 0.365) (Table S4; Fig. 3). A distinguished growth was observed in either medium that was expressed in OD values ranged from 0.189 to 0.467; being significantly higher for 1/10 CCM 30 compared to PBSW during the first and second steps of domestication. With further third step of domestication, progressive growth reported in PBSW culture medium and where differences were diminished among both tested culture media (Table S4; Fig. 3). As to growth conditions, and irrespective of plant compartments and culture media, a significant effect was reported for the longer incubation up to 8–12 days resulted in the highest growth and OD values ranged from 0.277 to 0.614 (Table S4; Fig. 3).

As to growth velocity, the standard 1/10 CCM 30 culture medium gave the shortest doubling time during the first step of in vitro domestication; being shortest for the endo-phyllosphere (26.6 h.) compared to the endo-rhizosphere (34.1 h). The corresponding values for PBSW culture medium were longer (40.1 and 36.6 h). With further steps of domestication, the doubling time progressively decreased. Efficiently, the third step of in vitro domestication shortened the doubling time of endo-phyllosphere microbiota down to 22.3 and 23.9 h in PBSW and 1/10 CCM 30 culture media respectively. The corresponding values for the endo-rhizosphere were somewhat higher and approached 25.13 and 25.12 h in PBSW and 1/10 CCM 30 culture media respectively (Fig. 3).

Up-scaling of in vitro domesticated biomass production

To up-scale the produced biomass of in vitro domesticated microbiota of the endo-rhizosphere in PBSW culture medium, a number of growth conditions were tested. This included: a) enriching the nutrients' concentration by increasing the volume of plant broth used for the preparation of batch culture media up to > 50—200 ml plant broth / L culture medium; b) various inoculum sizes (10 and 20% of the final volume of batch cultures %, v/v); c) different incubation temperatures (25 and 30 ℃) and intermittent/

Table 1 Log CFUs of culturable bacteria of endo-rhizosphere and endo-phyllosphere of *Salicornia europaea* L developed on 1/10 CCM30 and PBSW: One- and two- way ANOVA analyses

Factor (A) incubation time (days)		
5	$5.41^{b*} \pm 0.044$	
8	$5.59^{a} \pm 0.044$	
12	$5.69^{a} \pm 0.044$	
LSD (***P***-value ≤ 0.05)=0.18		
Factor (B) plant sphere		
Endo-rhizosphere	$6.47^{a} \pm 0.036$	
Endo-phyllosphere	$4.65^{b} \pm 0.036$	
LSD (***P***-value ≤ 0.05) = 0.15		
Factor (C) culture medium		
1/10 CCM30	$5.02^{b} \pm 0.036$	
PBSW	$6.11^{a} \pm 0.036$	
LSD (***P***-value ≤ 0.05) = 0.15		
Two-way interaction (B X C)		
Culture media	**Endo-rhizosphere**	**Endo-phyllosphere**
1/10 CCM30	$6.19^{b} \pm 0.051$	$3.85^{d} \pm 0.051$
PBSW	$6.76^{a} \pm 0.051$	$5.45^{c} \pm 0.051$
LSD (***P***-value ≤ 0.05) =0.21		

Bacterial counts were calculated on the basis of oven dry weight (70 °C)

Each figure represents the average of two biological replicates and average of CFUs developed on four replicates of agar plates

*Means followed by the same letter are not significantly different ($p < 0.05$)

1/10 CCM30; standard chemically-synthetic N-deficient combined carbon sources medium (3% w/v NaCl)

PBSW, plant broth-based-seawater culture medium

continuous shaking of prepared batch cultures. Results indicated that the higher the plant concentrations the better the measured bacterial growth, in terms of higher OD (1.119) and shorter DT (14–15 h) values. Additional positive and significant effects on the growth were reported with the use of inoculum volume of 10% (v/v) and intermittent shaking (Fig. 4). Differences among the incubation temperatures of 25 and 30 C were insignificant (Fig. S2).

Velocity of growth under such culturing conditions was evaluated in terms of the measured DT values. Taking into consideration ANOVA analyses of interacted treatments, the shortest doubling time of 10.7 h was reported for batch culture prepared using the higher plant broth concentration of 200 ml plant broth /L culture medium, specifically under the growth conditions of using the inoculum size of 10%, with continuous shaking and incubation at 25℃ (Fig. 4).

The measurements of pH and EC in prepared batch cultures for endo-rhizosphere and endo-phyllosphere of tested plant were monitored throughout the in vitro domestication steps. As to pH, it appeared that the plant broth-based sea water culture medium is having a buffering capacity that maintained pH values around pH 7.0. In contrast, pH values for batch cultures prepared from the chemically-synthetic 1/10 CCM 30 progressively decreased down to values < pH 6.0 by longer incubation. As to EC, no specific trend was observed amid the successive and good growth of microbiota of either plant compartments. (Table S4; Fig. S3). With trials of up-scaled biomass production, a somewhat similar trend was observed, where significant effects on pH and EC values were only attributed to longer incubation (Table S5).

Analysis of culture dependant/independent community composition of both intact *S. europaea* compartments and resulted in vitro-domesticated batch cultures

DGGE fingerprinting of the 16S rRNA PCR amplicons recovered from in vitro domesticated culturable

Table 2 Log numbers of CFUs [data are log means ± standard error (SE), n =4] of culturable microbiota of endo-phyllosphere developed on 1/10 CCM30 and PBSW culture media, and bacterial quantification using real-time PCR of S. europaea shoot. The mean value of qPCR cell numbers (obtained indirectly assuming that the average 16S rDNA copy number per cell is 3.6) is log 7.11±0.09 g−1 shoot dry weight obtained for ten replicates. Statistically significant differences are indicated by different letters (P value ≤ 0.05, n =14)

Culture media	**1/10 CCM30**	**PBSW**
Log CFUs g^{-1} DW	**3.85 ±0.051[b]**	**5.45 ±0.051[a]**
% culturability	**0.05%**	**2.19%**
Comparison of CFUs counts and morphology developed on agar plates of either culture media; using the same dilution of mother plates (10^{-2})		

PBSW, plant broth-based sea water culture medium

1/10 CCM30; standard chemically-synthetic N-deficient combined carbon sources medium (3% w/v NaCl)

communities, developed during domestication (steps 1 and 3), aimed at checking possible differential enrichment of endophytic bacteria developed in tested culture media compared to the culture independent samples extracted from intact roots and shoots, known as the original mother cultures. DGGE analysis resulted in clear banding patterns (Fig. S1), and cluster analyses of produced DGGE bands of bacterial endophytes of both plant compartments are illustrated in Figs.(5) and (S4). Based on the analysis of distance scores, the constructed dendrogram of endo-phyllosphere is shown in Fig. (5). Setting a cluster cutoff value of 0.6 resulted in two main different clusters. Among the first and main cluster, mother original cultures sub-clustered at Cutoff Value of 0.75 away from all in vitro domesticated steps. Further sub-clusters were separated at cutoff 0.8 where the majority of PBSW cultures distanced away from those of 1/10 CCM 30 culture medium and closer to mother cluster. No specific trend was observed among domestication steps in either medium. With endo-rhizosphere, two main clusters were separated at Cutoff Value of 0.75. At Cutoff Value of 0.9, the first cluster was further sub-clustered down to the mother original cultures closer to in vitro domesticated batch cultures of domestication step 3 of PBSW culture medium. The majority of the remaining sub-clusters of domesticated cultures of 1/10 CCM 30 medium were clustered further away (Fig. S4).

Diversity of bacterial isolates representing the culturable community of bacterial endophytes in *S. europaea* compartments

Representative colonies developed under various culture conditions were single-colony isolated and further sub-cultured. For endo-phyllosphere, a total of 535 colonies were sub-cultured, representing culturable community in plant mother cultures (188) and domesticated batch culture step 3 (347), developed on either 1/10 CCM 30 (289) or PBSW (246) culture medium. The corresponding number of colonies for

Table 3 Log CFUs of culturable bacteria developed in *in-vitro* domesticated batch cultures (step 3) prepared for endo-rhizosphere and endo-phyllosphere of *Salicornia europaea* L, and developed on 1/10 CCM30 and PBSW: One- and two- way ANOVA analyses

Factor (A) incubation time (days)		
4	$6.84^{c*} \pm 0.018$	
8	$7.14^{b} \pm 0.018$	
12	$7.24^{a} \pm 0.018$	
LSD (***P***-value ≤ 0.05) = 0.0247		
Factor (B) plant sphere		
Endo-rhizosphere	$7.07^{a} \pm 0.015$	
Endo-phyllosphere	$7.08^{a} \pm 0.015$	
LSD (***P***-value ≤ 0.05) = 0.0203		
Factor (C) culture medium		
1/10 CCM30	$6.87^{b} \pm 0.015$	
PBSW	$7.28^{a} \pm 0.015$	
LSD (*P*-value ≤ 0.05) = 0.0203		
Two-way interaction (B X C)		
Culture media	**Endo-rhizosphere**	**Endo-phyllosphere**
1/10 CCM30	$6.85^{b} \pm 0.021$	$6.90^{b} \pm 0.021$
PBSW	$7.29^{a} \pm 0.021$	$7.26^{a} \pm 0.021$
LSD (***P***-value ≤ 0.05) = 0.028		

Each figure represents the average of two biological replicates and average of CFUs developed on four replicates of Agar plates

*Means followed by the same letter are not significantly different ($p < 0.05$)

1/10 CCM30; standard chemically-synthetic N-deficient combined carbon sources medium (3% w/v NaCl)

PBSW; plant broth-based-seawater culture medium

endo-rhizosphere was 548; representing mother cultures (245), and domesticated batch culture step 3 (303) developed in 1/10 CCM 30 (254) and PBSW (294) culture media (Table S2). The successfully sub-cultured isolates were further tested for their growth preference on PBSW culture medium as well as tolerance to higher salt concentrations. Finally, a total of 154 isolates were secured and successfully 16S rRNA gene sequenced for various plant compartments, culture conditions and salt stresses. They represented the direct isolation from mother cultures, i.e. intact roots (endo-rhizosphere, 46) and shoots (endo-phyllosphere, 40 isolates), and domesticated batch cultures (68 isolates).

In general, the holobiont of the tested host plant was dominated by representatives of 3 major phyla: Pseudomonadota (73%) Bacillota (21%), Actinomycetota (6%) (Fig. 6). The present genera were dominated by the core genera *Halomonas* (representing > 70% of the culturable isolates) followed by *Oceanobacillus, Kocuria, Marinococcus, Metabacillus,* and *Bacillus*. The minor/satellite dwellers belonged to 9 genera of *Mixta, Gracilibacillus, Planomicrobium, Marinimicrobium, Salinicoccus, Zhihengliuella, Nesterenkonia, Kushneria,* and *Marinobacter* (Table S6; Fig. 6).

Isolates representing the original mother cultures, i.e. intact endo-rhizosphere/endo-phyllosphere of *S. europaea*

More than 35 isolates showed preference of growth in the plant broth-based culture medium under sea water salinity (*ca*. 3% salt) and represented both plant compartments. A total of 12 genera detected, 4 were common among endo-rhizosphere and endo-phyllosphere, namely**,** *Bacillus, Halomonas, Kocuria,* and *Oceanobacillus*. Further, a number of genera were confined to either endo-phyllosphere (5 genera*; Gracilibacillus, Marinococcus, Metabacillus, Mixta,* and *Planomicrobium*) or endo-rhizosphere (3 genera, *Marinimicrobium, Salinicoccus,* and *Zhihengliuella*) (Fig. 7A, B, C, D).

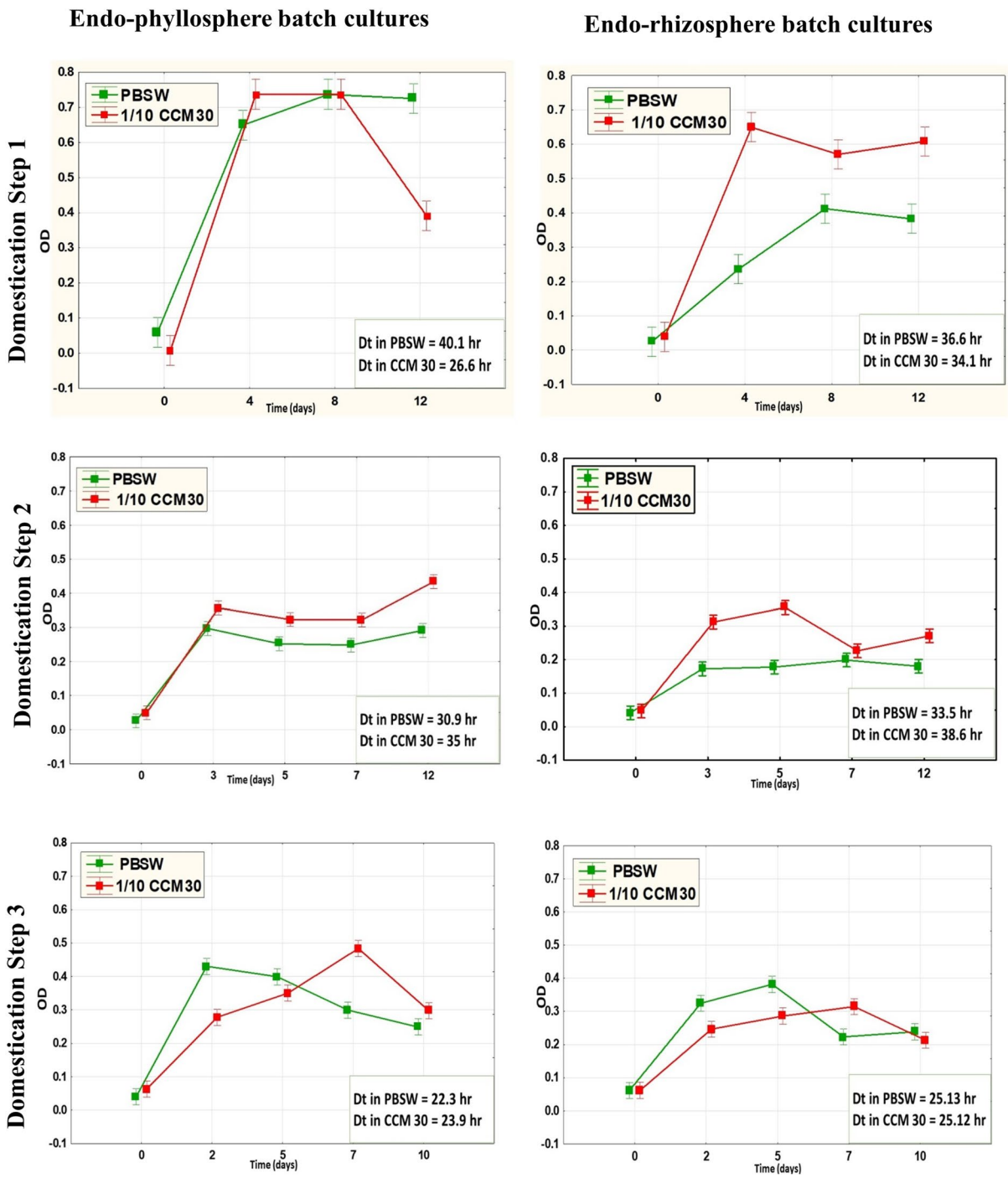
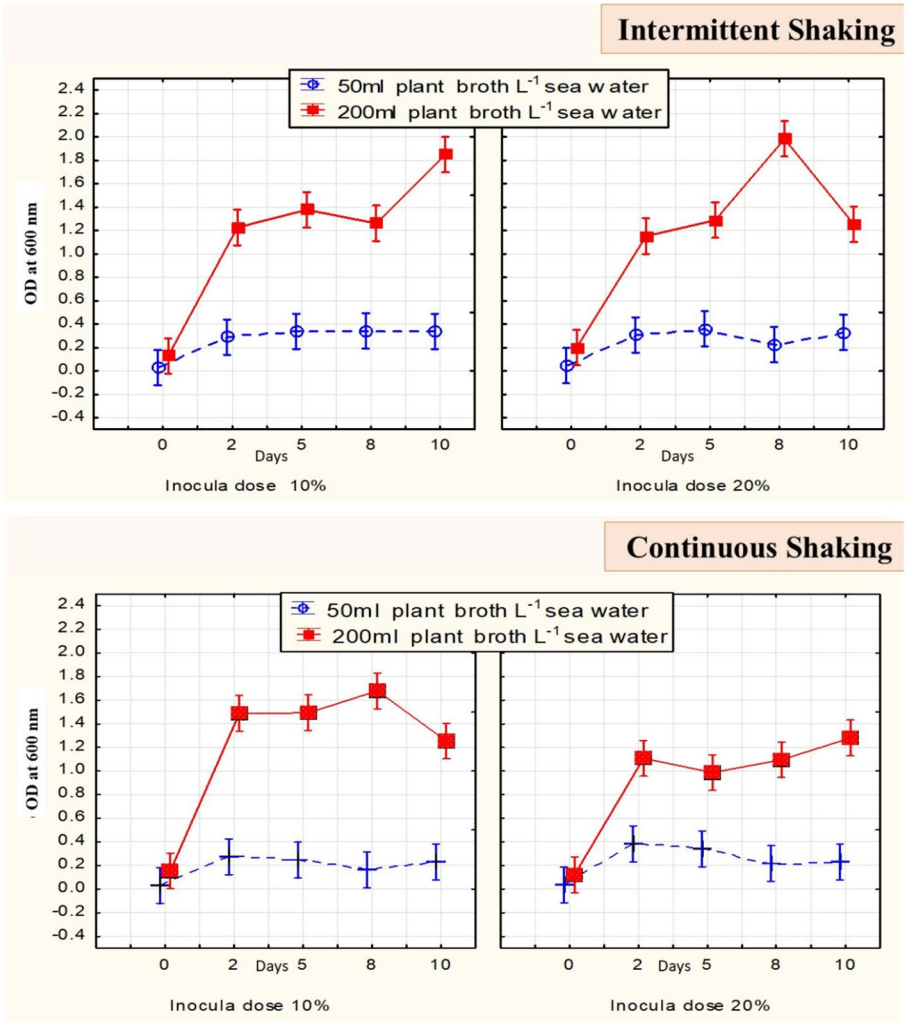


Fig. 3 Growth of halophyte microbiota of *S. europaea* in the prepared batch cultures of the three successively- domesticated steps of both endo-rhizosphere and endo-phyllosphere, using plant broth-based-sea water culture medium (PBSW;25 ml, 50 ml L^{-1}) and salted standard chemically-synthetic N-deficient combined carbon sources medium (3% w/v NaCl) (1/10 CCM 30): Optical density (OD) and doubling time (Dt, hr.)

A



B

Doubling times								
	Intermittent shaking				Continuous shaking			
Incubation temperature	25°C		30°C		25°C		30°C	
Inocula dose	10%	20%	10%	20%	10%	20%	10%	20%
50 ml L^{-1}	31.4^{a}	21.2^{cb}	18.4^{cd}	17.8^{cde}	18.4^{cd}	17.9^{cde}	24^{b}	17.8^{cde}
200 ml L^{-1}	15.2^{ed}	15.2^{ed}	15.2^{de}	14.4^{def}	10.7^{f}	14.1^{def}	15.2^{de}	14^{ef}

C

OD_{600}								
Treatments	PBSW culture media concentration		Inoculation dose		Incubation temperature		Shaking	
	50ml	200ml	10%	20%	25°C	30°C	Continuous	Intermittent
	0.238^{b}	1.119^{a}	0.709^{a}	0.647^{b}	0.667^{a}	0.689^{a}	0.603^{b}	0.742^{a}
L.S.D. (P-value ≤ 0.05)	0.133		0.133		0.133		0.133	

◂**Fig. 4** The up-scaled biomass produced in PBSW culture medium: interaction of nutrient concentration (50 and 200 ml plant broth / L culture medium) with inoculum size (10 and 20% of the final volume of batch cultures) under intermittent and continuous shaking (**A**). inserted is ANOVA analyses of the respective doubling times (**B**) and optical density OD_{600} (**C**)

A total of 195 isolates were tested on higher salt concentrations (10 and 30% NaCl), and 51 isolates demonstrated excellent growth on 30% salt; i.e. exercising a true halophyte niche in salty environments (Fig. 7 C, D). They belonged to 7 genera as follows: *Halomonas* were common in both compartments, while the endo-phyllosphere was marked by the presence of *Kushneria*, *Marinococcus*, and *Oceanobacillus*; and endo-rhizosphere by *Kocuria*, *Marinobacter*, and *Nesterenkonia.* It is obvious that with higher salt concentration, a reduced diversity was observed. For endo-phyllosphere, the higher abundance was for *Oceanobacillus* compared to *Halomonas* in endo-rhizosphere (Fig. 8).

Isolates dominated the tested in vitro-domesticated batch cultures

Domestication of halophytic microbiota in successive batch cultures of plant broth-based sea water culture medium reduced the diversity of the culturable microbiota. For endo-phyllosphere, domestication resulted in the disappearance of taxa of Actinomycetota, and the continual growth of those of Bacillota and Pseudomonadota. With endo-rhizosphere, the effect was abrupt and sharp where all phyla disappeared except those belonging to Pseudomonadota (Fig. 9 and graphical abstract). At genera level, domestication resulted in the overwhelming presence of *Halomonas* in the endo-rhizosphere compartment, out of total of 9 genera present in the original mother culture. With endo-phyllosphere, 4 genera (*Bacillus, Gracilibacillus, Halomonas,* and *Oceanobacillus)* thrived in the in vitro domestication compared to 10 genera that were commonly present in the mother cultures.

Discussion

Revealing the functionality of plant–microbe interactions and factors involved in community assembly can lead to a better understanding of the plant as a meta-organism and how plants can benefit from their microbial partners (Brader et al. 2017; Lemanceau et al. 2017; Hardoim et al. 2015; Hacquard 2016). Hence, this intrigued the interest and efforts of studying halophyte communities, e.g. *Salicornia europaea*, which are common to salt-affected environments of Egypt and to delve into their associated microbiota. This particular and obligate halophyte is commonly found in coastal and inland salt marshes (Flowers and Colmer 2008; Hrynkiewicz et al. 2019), and geographically it is native to north and west Europe (POWO 2024), and now it is distributed in America, Asia, Africa, and Europe (Kadereit et al. 2007). The present study used *Salicornia europaea* prevailing in the salt- affected area of Lake Mariout, north-western part of the Mediterranean coast, Alexandria-Egypt; an environment that is under the salt stress of the Mediterranean Sea water. This particular halophyte possesses several physiological and bio-chemical traits that permit its growth and persistence in such marginal conditions (Cárdenas-Pérez et al. 2022). A part of its successful adaptation is attributed to the ability to establish effective association with endophytic microorganisms residing in its internal tissues which positively affect plant nutrition, health and hormonal balance (Hrynkiewicz et al. 2019; Mapelli et al. 2013; Piernik et al. 2017; Szymańska et al. 2016).

The main aim of this study was to apply strategies of plant microbiome culturomics (Elsawey et al. 2020; Mourad et al. 2018; Nemr et al. 2020, 2021; Sarhan et al. 2016; Youssef et al. 2016), via the use of the advanced plant-broth-based sea water culture medium (PBSW) (Saleh et al. 2017) in comparison to the chemically synthetic salted combined carbon sourced medium. Such culture medium combines both the rich nutrients of the tested host plant and the neighbouring sea water exercising the required salt stress that provides a compatible nutritional matrix that enhances culturability of salt- tolerant endophytic microbiota. Results indicated that in vitro culturability was very well improved and resulted in higher CFUs counts with the special use of such PBSW compared to the salted-standard chemically-synthetic culture medium, 1/10 CCM 30, with population increases approaching > 10 folds. One of the main reasons for the suitability of PBSW culture medium to widen the scope of culturing halophytic endophytes is its particular nutritional make up as indicated in

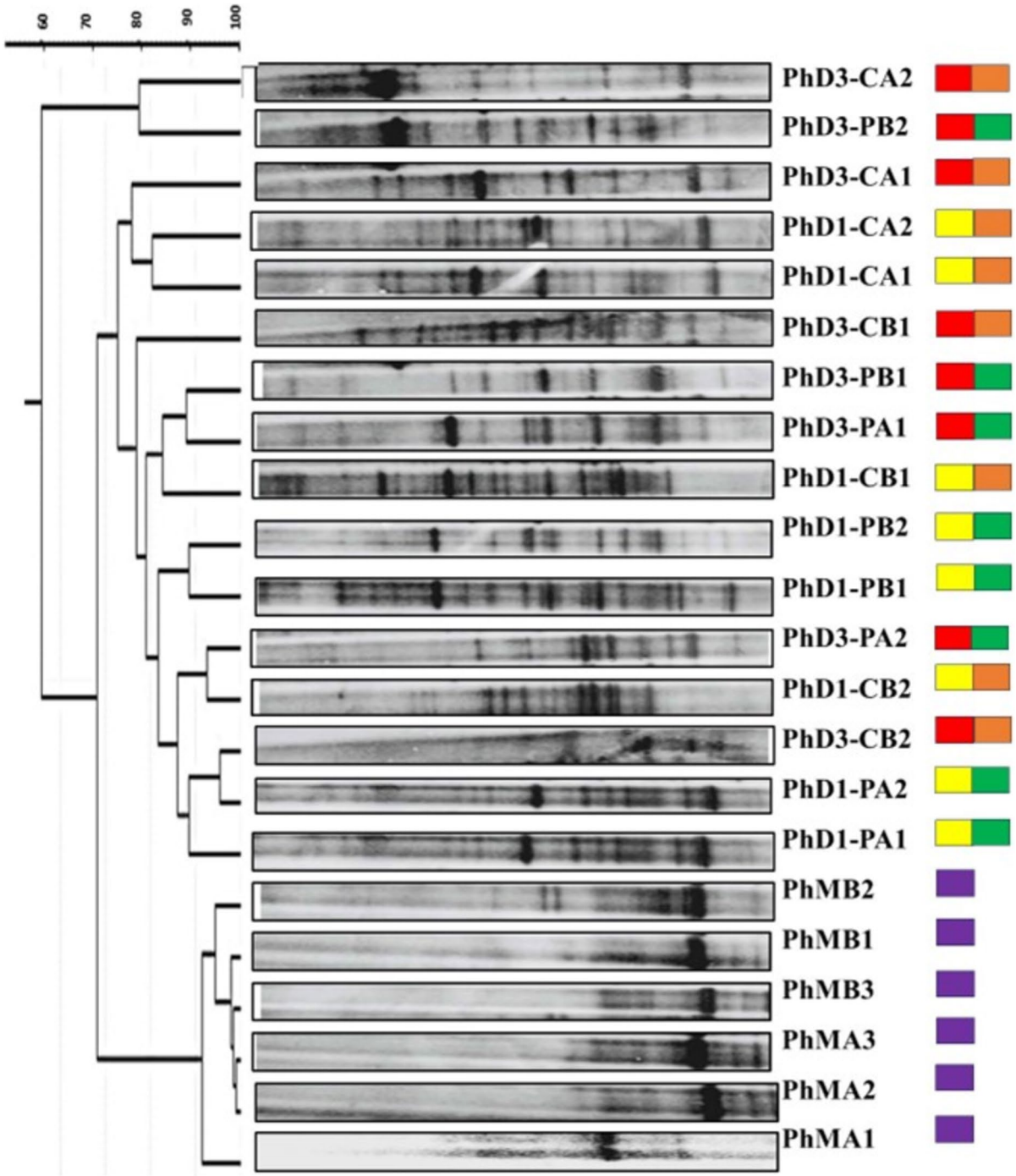


Fig. 5 DGGE bands obtained for endo-phyllosphere bacterial community. The analysis included culture independent samples of original mother cultures of shoot (PhM) samples; as well as culture-dependent communities developed in batch cultures of domestication step 1 (D1) and step 3 (D3) of both tested culture media of plant broth-based sea water culture medium (P),and chemically synthetic culture medium (C); including two biological replicates (**A**, **B**)

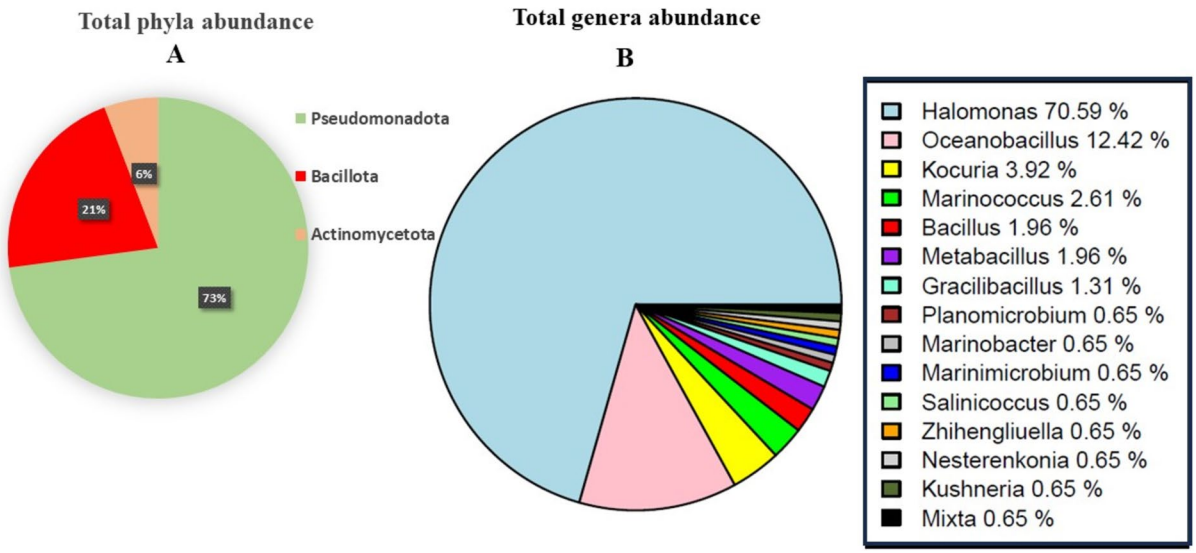


Fig. 6 Over all taxonomic position of total culturable isolates developed on PBSW, and representing various plant compartments and growth conditions: at phyla level (**A**) and genera level (**B**)

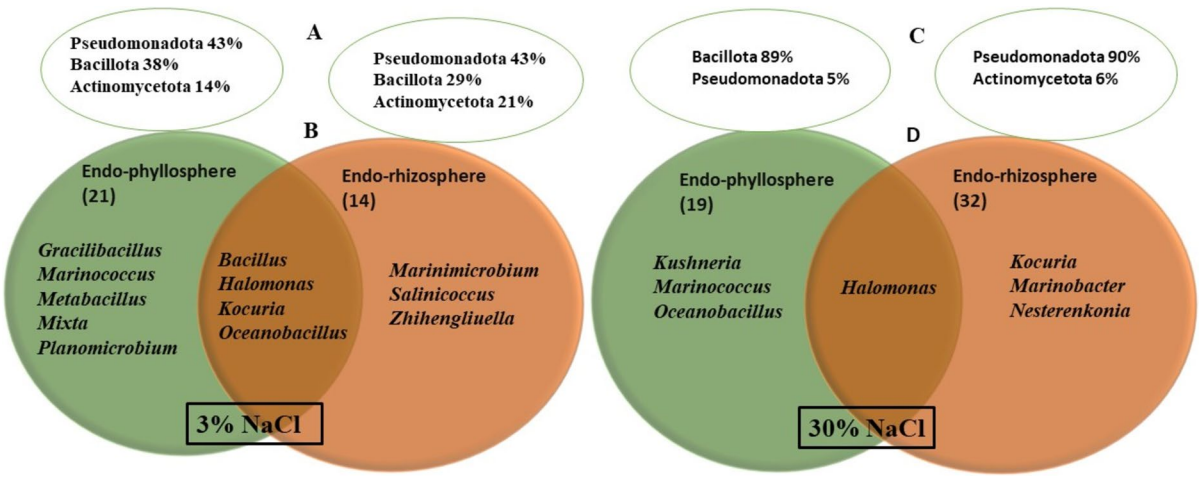


Fig. 7 Detected phyla (**A**, **C**) and genera (**B**, **D**) of culturable taxa developed on PBSW, and represented both plant compartments at growth conditions of 3% NaCl (**A**, **B**) and 30% NaCl (**C**, **D**)

Table (S7). Similar to the unique nutritional information presented in a number of publications confirming the richness of plant-derived culture media with respect of C and N compounds, macro-/micronutrients, vitamins, and co-factors (Saleh et al. 2017; Mourad et al. 2018; Elsawey et al. 2020; Nemr et al. 2020, 2021). Statistically significant differences were not only attributed to the single effects of the culture media type but also to the plant compartment. The endo-rhizosphere supported higher population density compared to endo-phyllosphere. A common finding that goes along with various reports in the literature (Daanaa et al. 2020; Elsawey et al. 2023; Nemr et al. 2020; Saleh et al. 2017; Sarhan et al. 2019). In fact, the rhizosphere is a distinguished ecological repertoire that is enriched by cosmopolitan cocktail and mosaic nutritional gradients under the effect of available plant residues, rhizodeposits, secretions, root

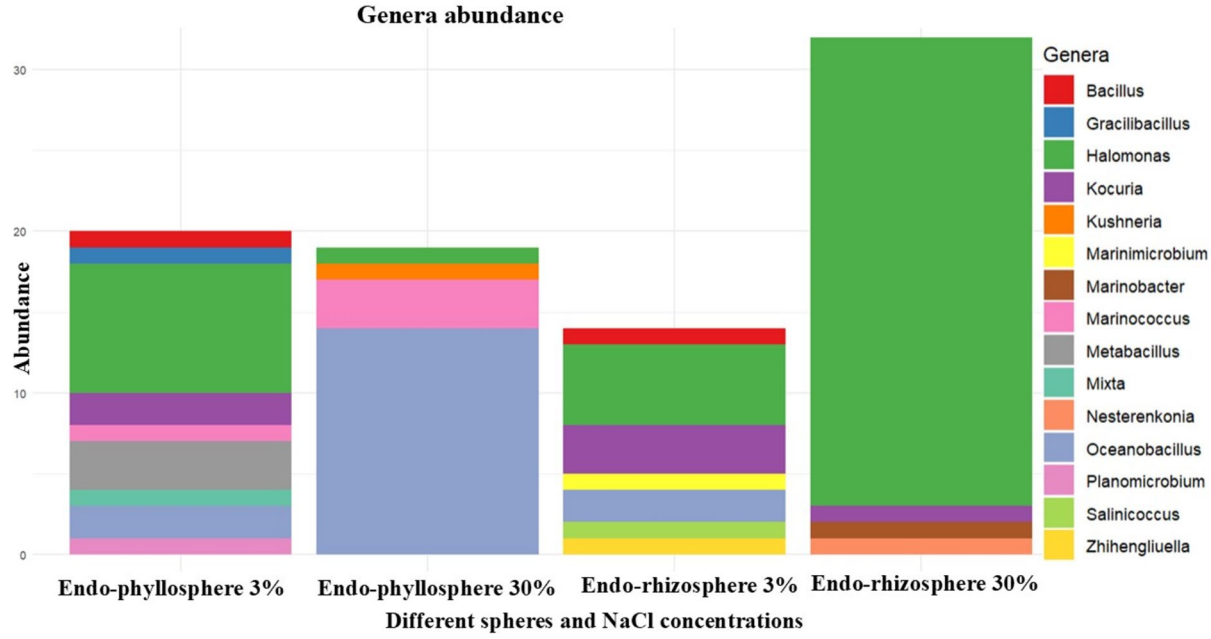


Fig. 8 Total abundance of culturable taxa (genera level) recovered from both plant compartments as affected by salt concentration in PBSW culture medium

mass, exudates, slough offs and root/biota respiration (Dakora and Phillips 2002; Sarhan et al. 2019; Willig et al. 2000).

The positive effect of using PBSW culture medium was extended to in vitro domestication in liquid batch cultures, supporting better development and higher

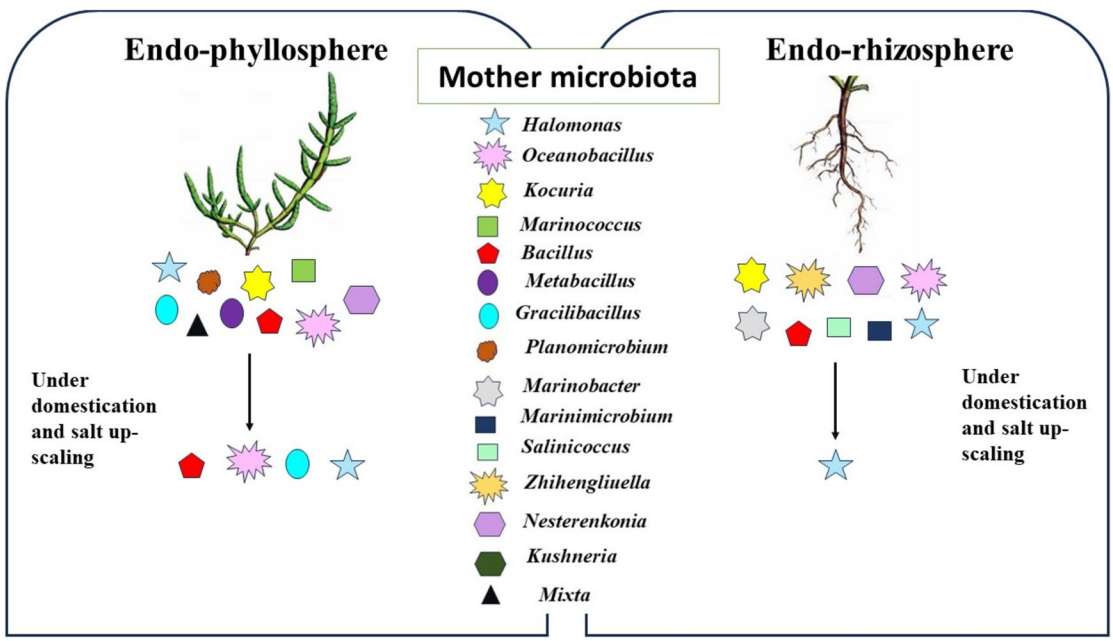


Fig. 9 Effect of in vitro domestication in PBSW batch cultures compared to intact (mother) halophyte microbiota of endo- rhizosphere and endo-phyllosphere at genera level

counts of CFUs compared to the salted-standard 1/10 CCM 30 culture medium.. Among the various methods employed to measure direct/ indirect bacterial growth (cell dry weight, cell optical density, cell turbidity, cell respiration, metabolic rate, and metabolites (Najafpour 2007), OD measurements and related DT values were periodically monitored. Initially, the lowest concentrations of plant broth (25–50 ml plant broth/L culture medium) were used in the preparation of liquid batch cultures. The idea of such low concentrations of nutrients is to allow the culturability of a wider range of halophyte microbiota, and to avoid stress of concentrated nutrients. This agrees with several reports indicating that dilutions of conventional culture media provided nutrients with appropriate concentrations required for the growth of colonies, including micro-colonies, and expanding the range of cultivable microbial species, in particular the fastidious groups (Elsawey et al. 2020; Sun et al. 2019). Initially and during the first and second domestication steps, the standard 1/10 CCM 30 culture medium supported denser growth and biomass production compared to PBSW culture medium. Such differences diminished by further domestication. Similarly, growth velocity in terms of doubling time (DT) was progressively decreased by increasing steps of in vitro domestication; and with the third step of domestication, DT values were comparable among the tested culture media. This is a clear indication of the progressive acclimatization of *Salicornia* microbiota under the designed in vitro domestication schemes. These findings align with previous reports that indicated that plant media can be as effective as standard/synthetic culture media for biomass production of various rhizobacteria in batch cultures (Daanaa et al. 2020; Hegazi et al. 2013; Youssef et al. 2016). Taking into consideration the limitations of batch culture studies that focus mostly on single organisms and being more suitable for high-throughput screening for industrial biomass production (Adamberg et al. 2020; Steensels et al. 2019).

To up-scale biomass production in the prepared batch cultures, a number of variables were applied. Among which was the nutrients' concentration by increasing the volume of plant broth used for the preparation of batch culture medium to 200 ml plant broth / L culture medium. OD measurements and DT values were significantly improved and supported higher biomass production. This is in addition to the significant effects of the use of inoculum volume of 10% (v/v) and intermittent shaking. Such inoculum effect has been observed with many organisms, often attributed to quorum sensing mechanisms (Gray and Smith 2005; Miller and Bassler 2001; Smith et al. 2004; Surette et al. 1999). Additionally, PBSW culture medium created more compatible growth conditions by having a buffering capacity that maintained pH values around 7.0, compared to the progressive decrease of values (< 6.0) of the standard 1/10 CCM 30 culture medium. This observation aligns with findings by Daanaa et al. (2020), where changes in pH were lower in the plant pellets-based culture medium compared to the standard yeast extract mannitol medium (YEM) for biomass production of rhizobia. Here, the authors also applied another approach to up-scale biomass production through supplements of agro-industrial wastes (molasses) as additional carbon source.

The microbiota of the endo-phyllosphere were progressively in vitro domesticated resulting in significantly higher OD values compared to the endo-rhizosphere. Such differences are likely attributed to the suitability of the used PBSW culture medium that is prepared from the vegetative parts of the plant, being more compatible for phyllosphere endophytes. Such culture medium compatibility was previously reported upon testing culture media based on various organs of the tested host plant, where endo-rhizosphere bacteria relatively thrived in root strips-based culture media and endo-phyllosphere bacteria preferred leaf strips-based culture medium (Nemr et al. 2021).

The effectiveness of the PBSW culture medium was further confirmed by quantifying the copy number of the bacterial 16S rRNA gene of *S. europaea* shoot by qPCR. The culture medium demonstrated significantly higher culturability, reaching up to 2.19% of the qPCR-calculated bacterial cell numbers, which is 44 times higher than the 0.05% reported for 1/10 CCM 30 medium. This goes along with the fact that more than 99% of the microbial population remains undiscovered to-date by culture-dependant techniques (Bodor et al. 2020; Locey et al. 2017), with only a small fraction being culturable using the current conventional culturing methods (Hofer 2018; Pedrós-Alió and Manrubia 2016; Rappé and Giovannoni 2003; Sarhan et al. 2019). This emphasizes the importance of on-going efforts for optimizing culture media and conditions to better reflect the actual

microbial diversity present in environmental samples (Bodor et al. 2020).

High-throughput techniques such as next-generation sequencing (NGS) are commonly used to assess the genetic diversity of microbial communities. However, PCR-DGGE (Muyzer et al. 1993) remains a widely utilized, cost-efficient and routine method in plant-soil ecosystem studies. Despite some limitations (Vischetti et al. 2020), recent optimizations have made the technique suitable for plant and soil DNA applications, and provide a comparative overview of microbial community composition when combined with culture-dependent microbiological approaches (Elsawey et al. 2023; Nemr et al. 2021; Sarhan et al. 2016; Vischetti et al. 2020). In the present study, the majority of in vitro-domesticated PBSW cultures formed a cluster distinct from 1/10 CCM 30 cultures, and were closer to the mother cluster. Such differences in DGGE patterns suggest a certain degree of culture media affinity and compatibility that influence community composition in designed in vitro domesticated batch cultures. Such effect is very much related to the nutritional makeup of the tested host plant used in the preparation of the plant broth-based seawater culture medium.

In the present study, the multiple representative bacterial isolates secured under various culture conditions were 16S rRNA gene sequenced and presented a wide diversity of culturable taxa. In general, *S. europaea* was dominated by representatives of three major phyla: Pseudomonadota (Proteobacteria) (73%), Bacillota (Firmicutes) (21%), and Actinomycetota (Actinobacteria) (6%). These findings agree well with previous studies on *Salicornia europaea* (Dragojević et al. 2023; Hrynkiewicz et al. 2019; Mapelli et al. 2013; Szymańska et al. 2016). In fact, Proteobacteria were found to be of a greater abundance among halophyte microbiota indicating their important functions in halophytes adaptation to saline habitats (Ahmed et al. 2018; Fidalgo et al. 2016; Furtado et al. 2019)**.** The fifteen detected genera were dominated by the core genera of *Halomonas* (representing > 70%), *Oceanobacillus (*> 12%*), Kocuria (*> 3%) and *Marinococcus (*> 2%*),* followed by *Metabacillus, Bacillus, Mixta, Gracilibacillus, Planomicrobium, Marinimicrobium, Salinicoccus, Zhihengliuella, Nesterenkonia, Kushneria,* and *Marinobacter.* Many of such genera are reported in literature to be of potential PGPB functions and to enhance plant tolerance to stresses and drought, e.g. production of IAA and ammonia, ARA and P/K solubilisation (Table S8). Among the detected fifteen genera, 7 were reported in literature having distinguished affinity to the presence of macro-/micro-elements in their halophytic environments. Besides Phosphorus, they showed distinctive affinity to higher concentrations of K, Ca, Na and Fe followed by Mg, Zn, Mn, Cu, Se and Co (Table S9). Previous reports on other halophytes common to the study area (Mariout Lake) documented as well the dominance of *Halomonas* in addition to *Kocuria*, and *Bacillus*, being active in PGP functions (Saleh et al. 2017). Consistent reports on the dominance of *Halomonas* underscore their adaptability to halophytes in challenging environments (Dragojević et al. 2023; Mapelli et al. 2013). This emphasizes the potential application of such halophytic genera for promoting plant growth under saline /drought conditions, providing a sustainable alternative to chemical fertilizers and pesticides.

We compared the culturable genera developed by the presented culturomics strategies to those reported in literature (Table 4). Among 15 genera recovered, five genera (*Gracilibacillus, Metabacillus, Mixta, Salinicoccus,* and *Zhihengliuella*) brought into cultivation for the first time from *S. europaea.* In addition, 3 genera (Marinobacter*, Marinimicrobium,* and *Planomicrobium*) that previously resolved by culture independent methods were brought into cultivation. Taking into consideration the culturable genera of other Salicornia species, 4 genera were not reported before (*Mixta, Marinimicrobium, Metabacillus,* and *Planomicrobium*) while the remaining 4 genera (*Gracilibacillus, Marinobacter, Salinicoccus,* and *Zhihengliuella*) were isolated from *S.ramosissima, S.rubra, S. strobilacea,* and *S. brachiata***,** using various culture media (Table S10).

In general, higher and differential diversity of detected genera was reported for the endo-phyllosphere (Fig. 10) compared to the endo-rhizosphere (Fig. S5). Among the 12 detected genera that showed preference of growth in the PBSW culture medium, 4 genera (*Bacillus*, *Halomonas*, *Kocuria* and *Oceanobacillus)* were common in both of endo-rhizosphere and endo-phyllosphere, suggesting their general predominance and adaptability among the plant's endophytic community (Compant et al. 2010). Additionally, 8 genera were confined to either the endo-phyllosphere (*Gracilibacillus*, *Marinococcus*, *Mixta*, *Metabacillus*, and *Planomicrobium*) or the endo-rhizosphere (*Marinimicrobium*, *Salinicoccus*, and *Zhihengliuella*).

Table 4 Genera of bacterial isolates cultured by the tested plant-broth-based sea water culture medium (PBSW) compared to those previously reported in literature (using culture-dependent and culture-independent methods) for *Salicornia europaea* microbiota

Bacterial phyla/ genera	Mother isolates (isolates that grow on PBSW 3% not 1/10 CCM30)		Mother isolates (PBSW 30% NaCl)		Domestication III (PBSW 30% NaCl)		Literature survey	
	Plant spheres						Culture – dependent	Culture – independent
	Endo-phyllo-sphere	Endo-rhizos-phere	Endo-phyllo-sphere	Endo-rhizos-phere	Endo-phyllo-sphere	Endo-rhizos-phere		
Pseudomonadota								
Halomonas	**Yes**	**Yes**	–	**Yes**	**Yes**	**Yes**	**Yes** (Dragojević et al. 2023) (Mapelli et al. 2013)	**Yes** (Mapelli et al. 2013) (Furtado et al. 2019)
Kushneria	–	–	**Yes**	–	–	–	**Yes** (Dragojević et al. 2023) (Mapelli et al. 2013) (Szymańska et al. 2016)	**Yes** (Furtado et al. 2019)
Marinimicrobium	–	**Yes**		–	–	–	–	**Yes** (Furtado et al. 2019)
Marinobacter	–	–	**Yes**	**Yes**	–	–	–	**Yes** (Furtado et al. 2019) (Ferreira et al. 2023a, 2023b)
Mixta	**Yes**	–	–	–	–	–	–	–
Actinomycetota								
Kocuria	**Yes**	**Yes**	–	**Yes**	–	–	**Yes** (Zhao et al. 2016)	
Nesterenkonia	–	–	–	**Yes**	–	–	**Yes** (Mapelli et al. 2013)	**Yes** (Mapelli et al. 2013)
Zhihengliuella				**Yes**			–	–
Bacillota								
Bacillus	**Yes**	–	–	–	–	–	**Yes** (Zhao et al. 2016) (Szymańska et al. 2016) (Ferreira et al. 2023a, 2023b) (Hrynkiewicz et al. 2019)	–
Gracilibacillus	**Yes**	–	–	–	–	–	–	–
Marinococcus	**Yes**	–	**Yes**	–	–	–	**Yes** (Mapelli et al. 2013)	–
Metabacillus	**Yes**	–	–	–	–	–	–	–
Oceanobacillus	**Yes**	–	**Yes**	–	–	–	**Yes** (Mapelli et al. 2013)	–
Planomicrobium	**Yes**	–	–	–	–	–	–	**Yes** (Mapelli et al. 2013)
Salinicoccus	-	**Yes**	–	–	–	–	–	–

Yes; reported, - ; not reported

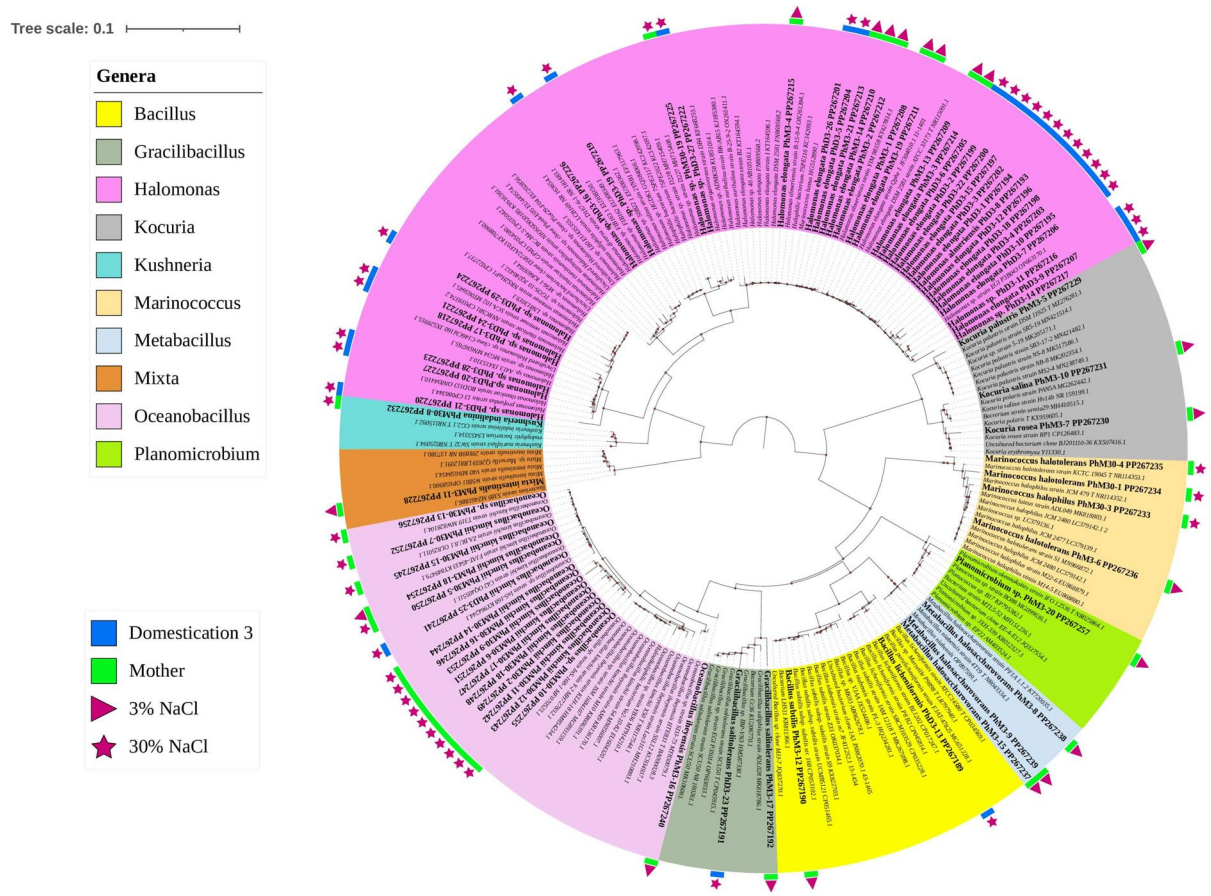


Fig. 10 Phylogenetic tree of 16S rRNA gene sequences of the 69 isolates representing endo-phyllosphere of *S. europaea*. The coloured labels indicate the taxonomic genera, growth conditions and culture media. Respective isolates identifier followed by their accession number are highlighted in bold and larger font style. In contrast, closely related strains are written in normal style, while type strains are indicated by a T before the accession number. The phylogenetic tree was interfered with neighbor-joining method. The bootstrap values for 1,000 replicates are adorning the branches as cycles

This pattern of compartmentalization reflects a certain degree of their specific adaptation to unique niches within the plant organs (Mapelli et al. 2013).

Salinity may act as an environmental filter that selectively favours microbes withstanding high salt concentrations, and shaping the microbial community composition of halophytes (Furtado et al. 2019). By increasing salt concentrations, less diversity was observed. Among the fifty-one isolates that showed tolerance to higher salt concentrations (30% NaCl), *Halomonas* were common to both plant compartments, while the endo-phyllosphere gave prominence to *Kushneria*, *Marinococcus*, and *Oceanobacillus,* and the endo-rhizosphere favoured *Kocuria*, *Marinobacter*, and *Nesterenkonia*. The high salt concentration supported the growth of such true halophytes which are known for their extreme halotolerance and ability to thrive in high-salinity environments (Meinzer et al. 2023; Soto-Padilla et al. 2018). This adaptability may be crucial for the survival and growth of *S. europaea* in saline conditions, demonstrating the potential of these isolates to be applied to bio-saline agriculture (Meinzer et al. 2023; Mukhtar et al. 2019a, b; Radhakrishnan et al. 2017).

In fact, in vitro domestication did not result in higher diversity of halophyte microbiota rather brought to a focus the potential true halophytes under the exercised higher salt concentrations (10–30%). At the genus level, domestication resulted in the overwhelming presence of *Halomonas* of the endo-rhizosphere

amongst the nine genera present in the original community of related mother cultures. Comparably, four genera (*Bacillus*, *Gracilibacillus, Halomonas*, and *Oceanobacillus*) flourished during *in-vitro* domestication of the endo-phyllosphere, out of the ten genera commonly present in the mother cultures (Fig. 9). This is a further indication on the compatibility of the used plant-broth-based sea water culture medium to microbiota residing the vegetative parts, the endo-phyllosphere, of *S. europaea.* In addition, the advanced in vitro domestication protocol creates an in situ *similis* environment for the growth and biomass production of such core genera under high-salinity conditions. In fact, the designed domestication steps exercised intense competition among existing taxa in each of the domestication steps, allowing for a "survival of the fittest" in the tailored-controlled environment (Steensels et al. 2019).

Conclusion

Pre-requisite to the introduction of microbiome-mediated strategies, especially in saline and drought-affected areas, it is imperative to explore and access the natural resources of the microbiota associated with halophytes. The advanced culturomics strategy based on PBSW culture medium created an in situ *similis* environment that significantly improved in vitro cultivation/domestication of the *S. europaea* microbiota, where the host plant-broth fed microbiota with innate/real time plant nutrients and sea water exercised natural salt stress. Compared to salted-standard culture medium, PBSW proved to be more compatible, enhance culturability, allow for greater exploration of bacterial diversity, and provide opportunities to culture previously unreported/unculturable microbiota. The tailored in vitro domestication strategy is applicable for recovering and identifying halophyte core microbiota, and further advanced to in vitro domesticate and propagate representative core microbiota, instead of laborious work of constructing consortia of single pure isolates, for SynCom applications.

Acknowledgements The authors acknowledge the financial support of the projects granted by STDF-Egypt (48635) and Cairo university (NGCS-plant microbiome biotechnologies, 2024). Nada acknowledges the generous travel support of Engineer Hamed Elshiaty of Shoura Group-Egypt and the granted research internship offered by IGZ, Großbeeren-Germany. The presented work is a continuation to the on-going joint research projects with German partners at Department of Plant Microbe Systems, Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany (IGZ).

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Funding Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). This work has been funded by the projects granted by STDF-Egypt (48635) and Cairo university (NGCS-plant microbiome biotechnologies, 2024).

Data Availability All data is included in the main text and the supplementary material.

Declarations

All authors have read and agreed to this version of the manuscript.

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