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Effect of site and phenological status on the potato bacterial rhizomicrobiota

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Abstract: Potato is the fourth major food crop in the world. Its cultivation can encounter problems, resulting in poor growth and reduced yield. Plant microbiota has shown the ability to increase growth and resistance. However, in the development of effective microbiota manipulation strategies, it is essential to know the effect of environmental variables on the microbiota composition and function. Here, we aimed to identify the differential impact of the site of cultivation and of plant growth stage on the potato rhizosphere microbiota. We performed 16S rRNA gene amplicon sequencing analysis of rhizospheric soil collected from potato plants grown in four sites in Central Italy, on two phenological stages. Rhizomicrobiota was mainly composed by members of phyla Acidobacteriota, Actinobacteriota, Chloroflexi and Proteobacteria and affected by both the site of cultivation and plant stages. However, cultivation sites overcome the effect of plant phenological stages. PiCRUST analysis suggested a high abundance of functions related to the biosynthesis of the siderophore enterobactin. The presence of site-specific taxa and functional profiling of the microbiota could be further exploited in long-term studies to evaluate the possibility to develop biomarkers for traceability of products and to exploit plant growth promoting abilities in the native potato microbiota.

Keywords: Potato; rhizosphere; microbiota; ecosystem functions

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1. Introduction

Potato is the fourth major food crops in the world and its cultivation in the field can encounters many problems, resulting in poor growth and reduced yield [1]. Besides plant genetics, crop yield is the result of a panoply of factors, including agronomic practices, soil, climatic conditions, and plant microbiota [2]. The plant microbiota, and especially the rhizospheric microbiota has been clearly related to crop yield and resistance to biotic and abiotic stresses [3–7]. As for many other plant species (see for instance [8]), the microbiota of potato rhizosphere has been shown to be strongly influenced by plant developmental stage [9–12], with the flowering stage generally having the highest diversity [9,10]. However, the effect of the site of cultivation is also relevant, since pedoclimatic conditions affect both soil microbiota composition and plant growth. In a survey of rhizosphere soil samples from potato grown at different altitudes [9], it was shown that richness (alpha diversity) was more affected by the site of sampling, as conditioned by different soil properties and climatic conditions, while the taxonomic composition was more significantly affected by the plant vegetation stage. When considering the exploitation of the plant microbiota for improving plant growth, in terms of preparation and formulation of bioinoculants, it is key to identify the core set of microbial taxa associated with the target plant species, i.e. those which are always present irrespective of environmental changes (e.g.

site of cultivation, growth stage, agronomic practices) and possibly of plant variety [13]. At the same time, the identification of taxa which can vary depending on the above-mentioned variables can allow to design tailored bioinoculant formulations, optimized under specific conditions [14,15], and also to define possible microbiological markers of, for instance, the locality of cultivation, to guarantee the traceability and enhance the area of the origin of the product [16–18]. This latter aspect is particularly relevant for Italy. In fact, although Italy is not one of the main potato producer [19], there are several traditional dishes that use local variety of potatoes but especially sites suitable for potato cultivation [20–22] in the hilly andmountain area of Central Apennines.

Consequently, it is highly relevant to perform an investigation on the potato-associated microbiota to provide additional data for a better understanding of the role phenological stages and cultivation sites have on potato microbiota diversity and consequently future rational development of bioinoculants and microbiome fingerprinting methods.

In this work, we aimed to identify the components of the bacterial rhizospheric microbiota of potato and their changes in relation to the site of cultivation and the plant growth stage on cv. Kennebec, a classical potato variety grown in the Italian central Apennine [23]. We answered this question by performing 16S rRNA gene amplicon sequencing analysis of rhizospheric soil collected from sites differing in pedoclimatic conditions and in two phenological stages, flowering, and harvesting.

2. Materials and Methods

2.1. Potato fields selection, sampling and physicochemical analysis

Four sampling sites cultivated with potato (*Solanum tuberosum* L.), cv. Kennebec, were selected in Tuscany (Central Italy) at different altitudes (Table 1). For the locality of Gavigno two sampling sites were considered to evaluate differences over a small distance (ca. 650 m distance). Fields were tilled and fertilized (Table 1) before sowing and no one received additional irrigation. For each sampling site, three soil samples were collected at the sowing at a depth of 15 cm and then mixed and analyzed to assess the main physical and chemical properties. The climatic conditions were computed for the entire growing cycle, from approximately early April 2020 (sowing) to late August 2020 (maturity). Temperature pattern was computed by the growing degree-day (GDD) following the method suggested by [24]:

$$\sum \text{GDD} = \frac{(\text{Tmax} + \text{Tmin})}{2} - \text{Tbase} \quad (1)$$

Where: ∑GDD is the sum of degree-days, Tmax is the maximum air temperature (°C), Tmin is the minimum air temperature (°C) and Tbase is the lower baseline temperature of the crop (°C). In this work, a Tbase of 4.4°C was used [25]. The amount of water supplied by rainfalls was calculated as cumulated monthly precipitations.

Sampling of soil was performed at flowering and harvesting stages from June to August 2020 under the guidance of the sampling campaign for the Crop Microbiome Survey (https://www.globalsustainableagriculture.org/the-crop-microbiome-survey/) [26], following the protocol defined at March 2020 (https://www.globalsustainableagriculture.org/wp-content/uploads/CropMicrobiome_March-2020.pdf). For each sampling sites and time, six samples were prepared, for a total of 48 samples (Table S1). Each sample was constituted by five individual plants at flowering stage or at harvesting stage. Selected plants were at a distance of at least 5 m from each other. After digging up the entire plant (10 cm radius around the plant, and down to 20 cm soil depth) and shaking off the plant roots, the soil remaining attached to the roots was detached with a sterile scalpel and considered as rhizospheric soil. Rhizospheric soils from each of the five plants were mixed, vortexed and stored at -80°C until DNA extraction. Physicochemical analysis of soils was performed as reported previously [27].

Table 1. Sampling sites analyzed in this study. The locality, geographical coordinates, and nitrogen (N), phosphoros (P) and potassium (K) fertilization rates are reported.

Locality	Geographical coordinates	Altitude	NPK Fertilization rate
Gavigno 1	44.068862° N, 11.093375° E	739 m	15-5-5 (500 g ha^{-1}).
Gavigno 2	44.062105° N, 11.095891° E	763 m	15-5-5 (500 Kg ha^{-1}).
Val di Chiana	43.412705° N, 11.784870° E	260 m	15-9-15 (100 kg ha^{-1})
Prato	43.860192° N, 11.054822° E	39 m	12-12-17 (350 Kg ha^{-1})

2.2. eDNA extraction and 16S rRNA gene amplicon sequencing

Environmental DNA (eDNA) was extracted from 500 mg of soil using the DNeasy PowerSoil Pro (Qiagen). From the extracted DNA, the bacterial V4 region of 16S rRNA genes was amplified as reported previously from 1 ng of total DNA [27]. Primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GACTACHVGGGTATCTAATCC-3′) were used [28]. Reactions were performed in a 25 μl total volume with KAPA HiFi HotStart ReadyMix, 1 μM each primer with 25 cycles with the following temperature profile: 30′′ 95°C, 30′′ 55°C, 30′′ 72°C. PCR products were sequenced in a single run using Illumina MiSeq technology with pair-end sequencing strategy and a MiSeq Reagent Kit v3 (Illumina, USA). Library preparation (Nextera XT, Illumina, USA) and demultiplexing were performed following Illumina's standard pipeline as previously reported [29].

2.3. Raw data processing, clustering and taxonomic assignment of reads

Illumina reads were trimmed using 'Trim Galore!' tool on Galaxy server (https://usegalaxy.org/). Paired-end sequences were clustered into Amplicon Sequence Variants (ASVs) following the DADA2 pipeline (version 1.16) [30]. Sequences were filtered and dereplicated using default parameters (MaxN=0, truncQ=2, rm.phix=TRUE and maxEE=2) to collapse identical reads into unique sequences and chimeras were removed. The taxonomy assignment was carried out by comparing our data against the SILVA NR99rel138 standard database of bacteria [31] using 'DECIPHER' R package (version 2.18.1) [32] as implementation of DADA2 (SSU version 138 available at: http://www2.decipher.codes/Downloads.html). Annotated ASVs count tables will be processed in Phyloseq package in R environment version 4.0.5 [33].

2.4. Diversity of the rhizosphere microbiota

The analysis of microbial communities was performed mainly following a previously published pipeline [27] through the 'Phyloseq' R package (version 1.34.0) [33]. For alpha diversity analysis, the Shannon and Simpson indices were calculated and plotted using the function 'diversity()' within 'microbiome' R package (version 1.12.0) [34]. Good's coverage and Evenness indices were calculated through the R functions 'goods()' and 'evenness()', respectively, within the 'microbiome' R package (version 1.12.0). Two evenness indices were computed (Camargo's index [35] and Pielou's index [36]) Rarefaction curves were generated using 'ggplot2' (version 3.3.3) [37] and 'ranacapa' (version 0.1.0) [38] R packages using the 'ggrare' function on phyloseq object. The 'ggplot2' R package (version 3.3.3) was used to generate relative abundance plots. Wilcoxon test for multiple comparisons of averages was performed on alpha diversity indices using the 'ggviolin()' and 'stat_compare_means()' within 'ggpubr' R package (version 0.4.0).

2.5. Effect of site and phenological stage on rhizosphere taxonomic composition

For ordination plots of phyloseq objects, a multivariate analysis based on Bray–Curtis distance and nonmetric multidimensional scaling (nMDS) ordination generated using the "ordinate" function was performed, and plots were generated using the "plot_ordination()"function within phyloseq package. Different community structures were analysed using permutational multivariate analysis of variance (PERMANOVA) performed using

the R packages 'ggplot2' (version 3.3.3), 'vegan' (version 2.5-7) and 'pairwise.Adonis' (version 0.0.1) using the functions 'adonis2()' and 'pairwise.adonis()', respectively.

Differential abundance analysis was carried out using the R package DeSeq2 v1.30.1 [39] in order to identify the ASVs/taxa differentially expressed along samples. The functions 'phyloseq_to_deseq2()', 'DESeq()' and 'results()' were used.

2.6. Functional inference on the rhizosphere microbiota

The metabolic pathway abundances and functional gene profiling were obtained by running the software PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, https://github.com/picrust/picrust2), as previously reported [27], using the Galaxy pipeline of Deng Lab [40]. Ranking of the most relevant pathways for varieties differentiation was conducted by running a SIMPER test using the function 'simper()' with the 'vegan' R package (version 2.5-7).

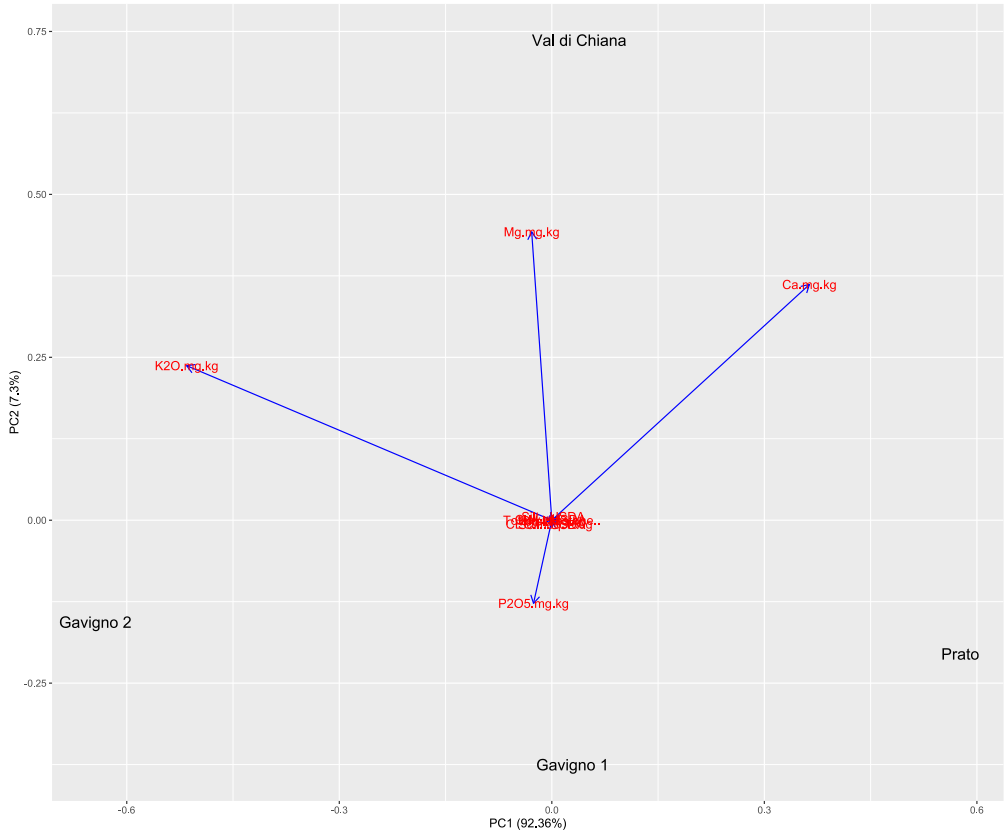
3. Results

3.1. Pedoclimatic conditions

Results of physical and chemical analysis of the soil are reported in Table S2. Based on the USDA soil textural triangle classification system [41], all the collected soil belongs to the silty loam class except Gavigno 2 which is classified as loam. The soils of Gavigno, being located in a wooded area, are richer in organic matter and have a more acid pH than the soils of the Val di Chiana (hilly area) and Prato (urban area). The soils mostly differed in P_2O_5, K_2O, Mg and Ca content and all samples showed different groupings when subjected to a Principal Component Analysis (PCA) performed on soil physicochemical parameters.

Climatic conditions are summarized in Figure S1. A large variability between months and sampling sites was observed both for temperature and precipitation. The monthly GDD varied from 172.5 (April) to 511.15 (August), from 219.3 to 606.25 and from 327.35 to 694.2 for Gavigno, Val di Chiana e Prato, respectively. As expected, Prato was the warmest site with 2686.25 GDD, followed by Val di Chiana (2211.65 GDD) and Gavigno (1806.4 GDD). Gavigno was the wettest sampling site, reaching a cumulated precipitation of 499 mm for the entire growing season, with maximum precipitation (149.2 mm) recorded in June and minimum precipitation (43.0 mm) recorded in July. The same trend was observed also in Val di Chiana with maximum (105.2 mm) and minimum (14.2 mm) rainfalls recorded in June and July, respectively. Prato was the driest site, reaching a cumulated precipitation of 239.2 mm from April to August, that is less than half compared to Gavigno. Unexpectedly, August was the wettest month (70.2 mm), while July was confirmed as the least rainy month (22 mm).

Figure 1. Influence of physicochemical composition of sampled localities. A Principal Component Analysis with a biplot is shown. The percentage of variance of the first two components is reported. Overlapped labels on the biplot indicates that they do not contribute to differences among localities.



3.2. Overall diversity of the rhizosphere bacterial microbiota

A total 3'760'646 16S rRNA reads were obtained and 2'721'248 (72 % of total reads) passed quality filtering (Table S1). After the clustering step, a total of 14'332 ASVs were obtained (Dataset 1). Rarefaction curves obtained from ASVs reached plateau for all samples (Figure S2), indicating a satisfactory survey of the bacterial diversity (Goods coverage, Table S3).

3.3. Location is the main driver of the taxonomic composition of potato bacterial rhizomicrobiota

Alpha diversity estimates were obtained and comparison between location and phenological stages were performed. Wilcoxon test indicated that Simpson index and both evenness indices showed different values among localities (Figure 2, Table S4). Moreover, the PERMANOVA reported that phenological stage and interaction between locality and stage significantly contribute to among samples differences (Table 2, Table S5).

Figure 2. Alpha diversity indices of the samples. A) Shannon index; B) Simpson index; C and D, Evenness (Camargo's and Pielou's indices, respectively). Lines and numbers above lines indicate P-value of contrasts (Wilcoxon test).

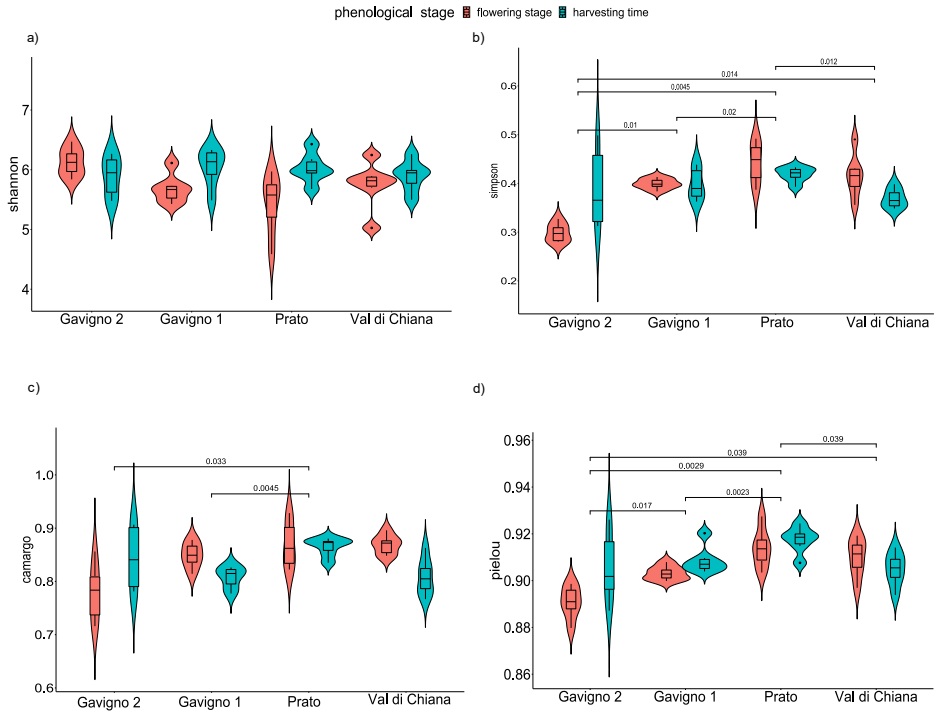
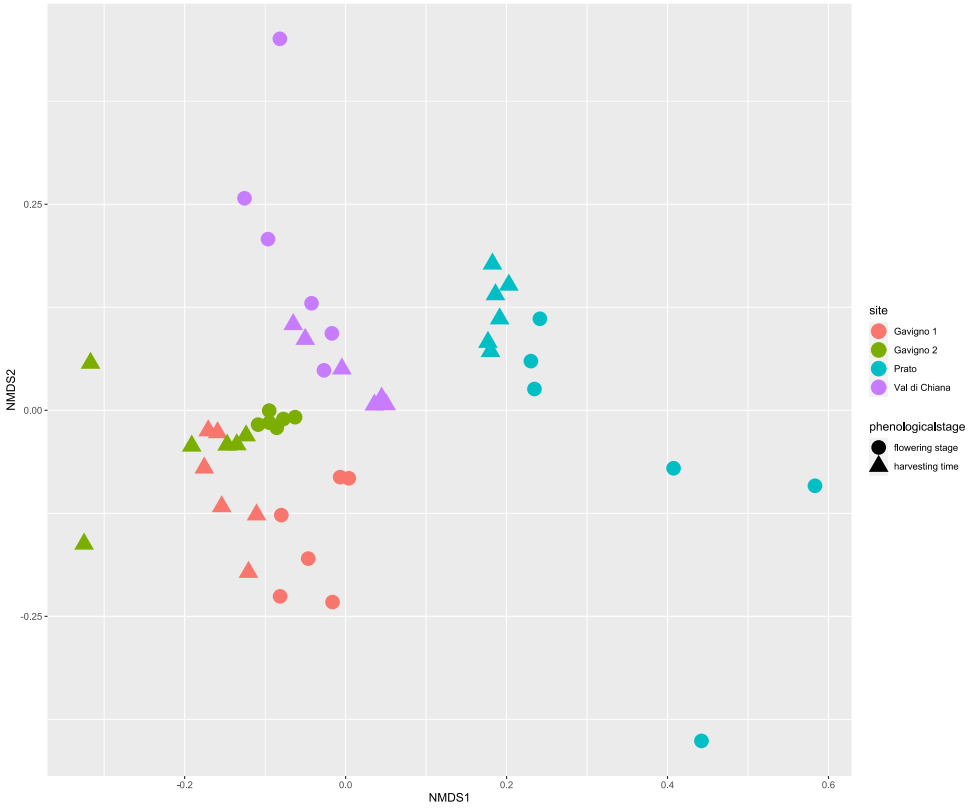


Table 2. Results of PERMANOVA on alpha diversity indices showing the contribution of the locality, phenological stage and their interaction. Pseudo F-statistic P-values are reported. n.s., not significant (P>0.05).

	Shannon		**Simpson**		**Pielou**		**Camargo**	
	F	***P-value***	**F**	***P-value***	**F**	***P-value***	**F**	***P-value***
Locality	15.064	n.s.	11.9274	0.001	6.2539	0.004	4.4182	0.011
Stage	52.131	0.018	0.4555	n.s.	2.7498	n.s.	0.6968	n.s.
Locality:stage	32.784	0.03	7.5625	0.002	4.7729	0.008	6.7443	0.001

The same pattern was observed for the taxonomic similarities among samples (Figure 3) with the two Gavigno set of samples (Gavigno 1 and Gavigno 2) grouping together, separated from Prato and Val di Chiana samples. On the same locality the two phenological stages showed a separation. A PERMANOVA test (Table S6) confirmed the high relevance of localities (sites, F=1.64, p<0.001) but confirmed also the contribution of phenological stage (F=1.17, p<0.01) and their interaction (F=1.19, p<0.001) in samples differentiation.

Figure 3. nonmetric Multidimensional Scaling of potato bacterial rhizomicrobiota. Colors indicate locations; circles, flowering stages; triangles, harvesting stages. Stress value = 0.17.



3.4. Taxonomic signatures of localities and phenological stages

Figure 4 reports the relative abundances of taxonomies at the phylum level. The analysis of the taxonomic composition revealed that the most abundant phyla were Acidobacteriota, Actinobacteriota, Chloroflexi and Proteobacteria.

In order to check the presence of the differential abundance of taxonomies with respect to localities and phenological stages, the package DESeq2 was used in the overall dataset. While no single ASV was found to be statistically different in abundance with respect to the phenological stage for all samples, 15 ASVs were found to be differentially distributed among localities, 9 of which were affiliated to the class Actinobacteria (Table S7), suggesting the presence of soil/locality-related microbiota signatures.

3.5. Functional potential of the potato rhizomicrobiota

Functional profiles were inferred from retrieved taxonomies in terms of metabolic pathways present in the bacterial taxa of the overall potato rhizomicrobiota. We identified a total of 434 pathways, distributed in all samples (Table S7). Using a SIMPER test, we identified nine and three pathways that significantly differentiate samples with respect to locality and phenological stage, respectively (Figure 5). The pathways included abilities related to the biosynthesis of molecules (ENTBACSYN-PWY, enterobactin biosynthesis), degradation of plant metabolites (PWY-5266, p-cymene degradation; PWY-5273, p-cumate degradation), anaerobic metabolism (PWY0-1533, methylphosphonate degradation; PWY-7007, methyl ketone biosynthesis; PWY-6876 (isopropanol biosynthesis)), carbon fixation (PWY-5392, reductive TCA cycle), and glycogen degradation (PWY-5941), pyrimidine biosynthesis (PWY-7187) and vitamin B12-related metabolism (adenosylcobalamin salvage from cobinamide II, PWY-6269). This latter was only found in relation to between phenological stages differences.

Figure 4. Relative abundances of bacterial phyla in the potato bacterial rhizomicrobiota. The four localities are shown separately. Samples from flowering and harvesting stages are indicated. See Table S1 for samples codes.

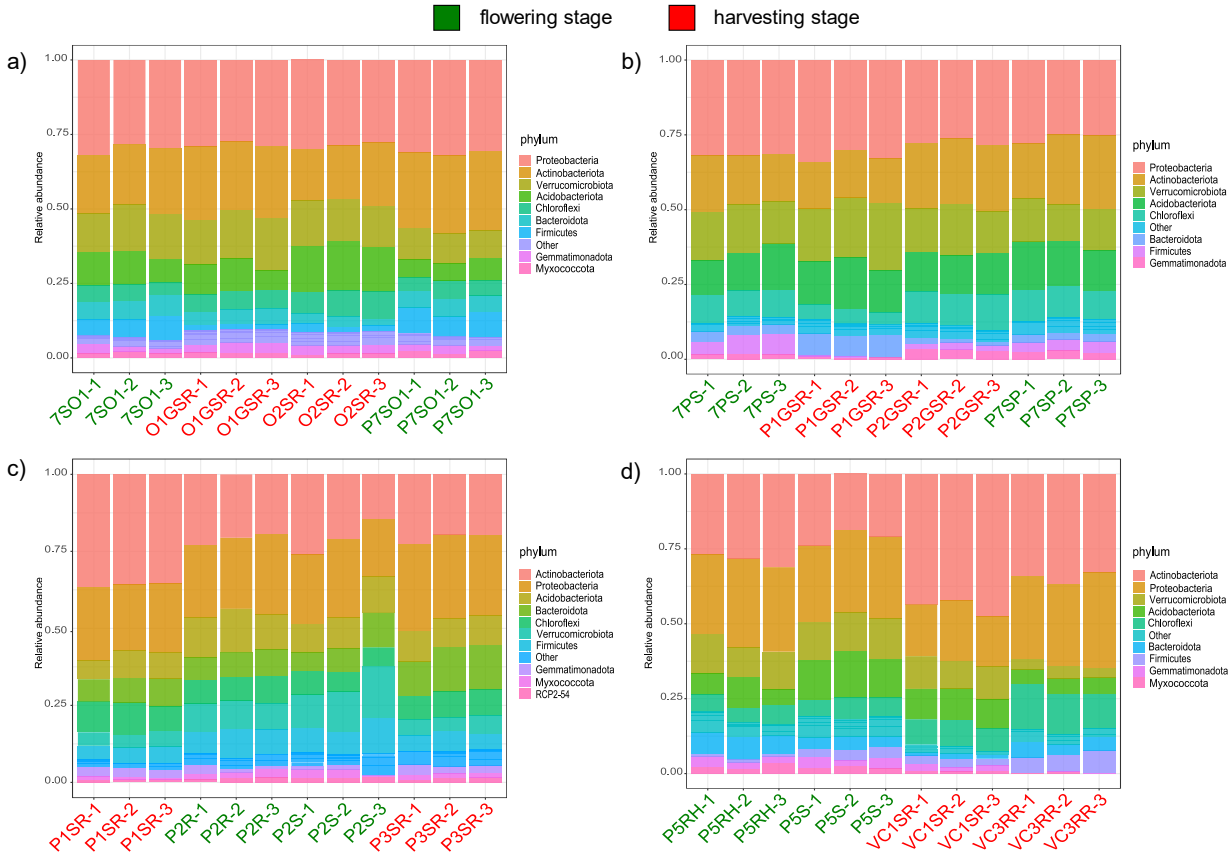
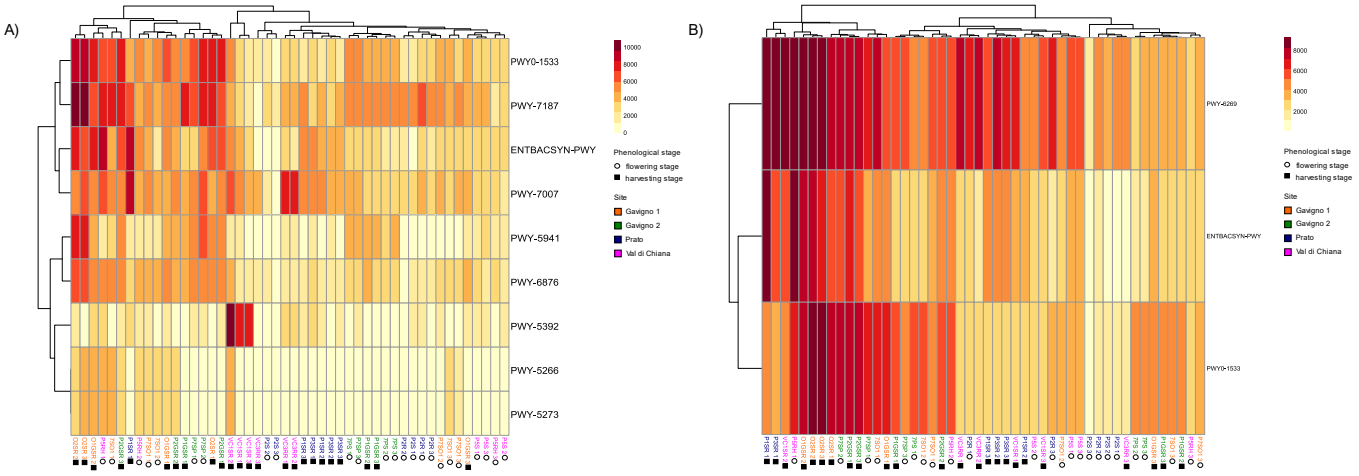


Figure 5. Metabolic pathways mainly contributing to differences among localities (A) and phenological stages (B). See Table S1 for samples codes.



4. Discussion

Here, we wanted to identify how much the rhizosphere microbiota of potato plants is impacted by the site of cultivation and if plant growth stage determines changes in the rhizomicrobiota. Obtained results clearly indicated that the overall diversity of the microbiota was principally affected by the pedoclimatic conditions of the site of cultivation, but the plant phenological stage also was shown to play a role in rhizomicrobiota diversity and taxonomic composition. Indeed, plant developmental stage is a relevant factor influencing rhizosphere microbiota [8]. In a previous work [9], conducted on low-input agriculture in the center of origin of potato (High Andes), alpha diversity was influenced by the sampling site, while effects of plant development on alpha diversity were less pronounced. This result is in agreement with evidence from our study, which was conducted using the same variety for all sample (cv. Kennebec). In fact, in our case-study also, alpha diversity mostly differs in relation to locality, and only to a lower extent in relation to plant phenological stages. Interestingly, in the same study [9], taxonomic diversity was significantly affected by the phenological stage, which perfectly matches with our results, indicating a stronger effect of phenological stage on the taxonomic structure of the microbiota, than that observed for alpha diversity. However, in case of taxonomic diversity, the main effect was still related to locality, deserving the possibility to identify microbiota-related markers of the site of cultivation, as previously observed for other plant species as barley, *Crocus* and orchids [16–18].

An abundant core microbiota, constituted by members of phyla Acidobacteriota, Actinobacteriota, Chloroflexi and Proteobacteria was found, irrespective of cultivation site and phenological stage. Acidobacteria, Actinobacteriota and Proteobacteria were found abundant in potato in other studies [9,10,42], indicating that differences in varieties, geographical locations, agronomic practices, and laboratory techniques (e.g. DNA extraction and sequencing methods) do not affect the overall taxonomic representation of bacterial potato rhizomicrobiota. Within these main phyla, Actinobacteriota were particularly interesting since the ASVs related to among-locality differences were taxonomically affiliated to this group. The presence of bacteria from the phylum Actinobacteriota (mainly from the genus *Streptomyces*) within these ASVs may suggests that production of secondary metabolites, including novel antibiotics could be an important function of the potato rhizomicrobiota. In fact, though a species from the genus *Streptomyces*, as *S. scabiei*, is the etiological agent of potato common scab disease [43], other strains from genus *Streptomyces* have shown important antagonistic activities and suppression of common scab disease [44]. We can envisage a future investigation on *Streptomyces* associated to potato rhizosphere and isolation of strains with relevant features in this context. Additionally, the presence of ASVs differentiating the localities also implies+ the possibility to develop DNA-based markers on such strains to allow the identification of cultivation sites and protection of local productions, a relevant aspect of Italian traditional food chain [22]. Finally, it is worth noting that the most abundant pathways represented after the PiCRUST analysis of the microbiota were those related to enterobactin biosynthesis and utilization of phenolic metabolites (p-cymene, p-cumate). Enterobactin is a well know siderophore, heling cells to chelate iron ions and transport them into the cell [45]. Siderophore production is a key functions of plant-associated bacteria [46,47] and in particular of growth-promoting bacteria [48], then highly relevant for bioinoculant formulations, where strains with important plant growth-promoting functions are combined. Competition for iron is also relevant for bacterial potato pathogens, as *Dickeya* spp. [49] and a relevant interplay between the plant and the microbiota exist for iron scavenging [50]. We can consequently look forward to the isolation of strains with such relevant function from the potato rhizomicrobiota as ingredients for future bioinoculant formulations helping potato with microelement nutrition [51]. The presence of abilities to degrade terpenes is also relevant and highlight the metabolic interaction between the host plant and the microbiota, as observed

in other systems [52–55]. Potatoes and cv. Kennebec in particular, are rich in terpene compounds [56], which are present on the skin of tubers and released during growth by root as root exudates.

In conclusion, this work allows to strengthen the importance of the site of cultivation for the potato microbiota and may suggests the presence of strains and functions, which can be further inspected for their possible use in improving potato cultivation in a sustainable way through bioinoculants, and in developing a microbiota-based fingerprinting of potato. However, we should be cautious to interpret present results on a general view. In fact, it would be interesting to know how stable this pattern of rhizomicrobiota is on different varieties and through the years, especially when farmers changed the fertilization and when we may have different temperatures and different amount of precipitation. Climatic conditions can influence bacterial and fungal pathogens, which, in turn, can influence the overall rhizomicrobiota. Indeed, since sites differed by climatic conditions also, additionally work should be done to evaluate the relationships on this small geographical scale, of climatic conditions, rhizomicrobiota and plant productivity to better integrate microbiota variation in an agronomic context.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

Author Contributions: Conceptualization, M.M. A.B., S.O., A.M. and L.C.; methodology, L.C.; A.B.; validation, A.B., M.M., L.C., C.F. and F.V.; formal analysis, L.C.; investigation, L.C., C.F., F.V., A.B.; resources, A.B., M.M. S.O. A.M.; data curation, A.M, L.C..; writing—original draft preparation, A.M, L.C.; writing—review and editing, A.B., M.M. S.O., A.M, L.C. and C.F.; supervision, A.M. and L.C.; project administration, A.M.; funding acquisition, A.M. S.O. M.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest

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