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PLANT TISSUE CULTURE

Plant growth-promoting rhizobacteria for *in vitro* and *ex vitro* performance enhancement of Apennines' Genepì (*Artemisia umbelliformis subsp. eriantha*), an endangered phytotherapeutic plant

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Abstract
Apennines genepì (*Artemisia umbelliformis* Lam. subsp. *eriantha*), is a high-altitude plant endangered by illegal and indiscriminate harvesting to produce the homonymous liqueur. Even if synthetic seeds represented a valid propagation technique to respond to this threat, this technology still requires methods to improve the low germination and plantlet development rates. The aim of the present study was to evaluate the suitability of the bacterial consortium formed by *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Burkholderia ambifaria*, and *Herbaspirillum seropedicae*, as an enrichment agent of Apennines Genepì synthetic seeds. To evaluate the effectiveness as an improving agent, the consortium was incorporated in the synthetic seeds, compared to a control, and followed until *ex vitro* cultivations. Plants were evaluated for their growth and development parameters, total phenolic and flavonoid contents, antioxidant properties, polyphenolic compounds concentrations, and volatile fractions. The results demonstrated that the investigated consortium is a good candidate as an enrichment agent in synthetic seed technology. The rhizobacterial presence induced an improved response in plant propagation and the acclimatization process. Plant quality was also enhanced, with an increased accumulation of plant secondary metabolites and higher antioxidant capacity. The investigated bacterial consortium is a good candidate as an enrichment agent in synthetic seed technology. Plant clones can be translocated for species conservation and also commercialized to reduce the illegal and undiscerning collections.

Keywords *In vitro* propagation · Synthetic seeds · Rhizobacterial plant growth promotion · Polyphenols · Volatile compounds · Antioxidants

Introduction

The disappearance of high altitude plants represents one of the current environmental threats of public and scientific concern. Several Central Apennines vascular plants are affected by this endangerment, mainly due to climate changes (Stanisci *et al.* 2005). The rainfall reduction, rare snowfalls, decrease in snow coverage, and increase in temperatures, led to the rarefaction of high-altitude species, with a gradual long-term transformation towards more drought-tolerant plants (Petriccione 2005; Thuiller 2007).

Artemisia umbelliformis Lam. subsp. *eriantha* (Apennines' Genepì), is a sub-endemic perennial plant that lives on limestone ravines fronting North above 2200 m, which has valuable ecological and phytotherapeutic properties (Mucciarelli *et al.* 1995; Pace *et al.* 2004; Rubiolo *et al.* 2009). It is an aromatic plant characterized by small size (10–15 cm stems) and inflorescences organized in a spike with small yellow flower heads (Fasciani *et al.* 2017). This plant, which is protected by an Abruzzo Regional Law, has been effectively

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micropropagated and acclimatized to *ex vitro* conditions (Pace *et al.* 2004). However, its existence is still threatened by illegal and indiscriminate harvesting to produce the homonymous liqueur (Pace *et al.* 2010).

To counteract plant extinctions, human-mediated translocations could help to manage endangered species conservation (IUCNS 2013; Reiter *et al.* 2018). Among conservation techniques, synthetic seed technology is a valid method to propagate and conserve clones (Gopinath *et al.* 2015) to decrease transfer and out of season subculture requirements (Rihan *et al.* 2017). Nevertheless, this technology requires modification to improve low germination and plantlet development rates (Javed *et al.* 2017; Magray *et al.* 2017). Several implementation agents can be employed, including nutrients, antibiotics, pesticides, and bacteria (Sahoo *et al.* 2012).

Many bacteria that belong to the plant growth-promoting rhizobacteria (PGPR) group, can improve plant germination and development, through mineral solubilization, nitrogen-fixation, and production of auxins, cytokinins, gibberellins, siderophores, and enzymes (Glick & Bashan 1997; Pérez-Montaño *et al.* 2014; Lamont and Pérez-Fernández 2016). Bacteria associated with this group have already been used in plant propagation, as improving agents in micropropagated *Musa acuminata* L. acclimatization and hardening (Suada *et al.* 2015), and as biotic elicitors of tropane alkaloid production in *Datura* spp. transgenic hairy roots (Moussous *et al.* 2018). In addition, PGPR volatile compounds were demonstrated to improve callus organogenesis in *Nicotiana tabacum* L. (Gopinath *et al.* 2015), and were effectively incorporated in alginate beads as inoculant carriers (Bashan *et al.* 2002). Strains that belong to *Azospirillium*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Enterobacter*, *Gluconacetobacter*, and *Herbaspirillum* genera, are the most commonly used PGPR, due to their recognized growth-promoting activities (Pagnani *et al.* 2018), and their ability to associate with almost all plant species (Rosenblueth & Martínez-Romero 2006). The consortium of *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Burkholderia ambifaria*, and *Herbaspirillum seropedicae*, isolated in association with crop plants (Yamada *et al.* 1997; Baldani *et al.* 1986; Coenye *et al.* 2001), demonstrated the ability to fix nitrogen and stimulate plant growth through the production of plant-growth promoting substances (Botta *et al.* 2013; Pagnani *et al.* 2018). The positive effects of this consortium have been established in *Lycopersicon esculentum* L. (Botta *et al.* 2013), in which treated plants recorded the best plant growth and development. The same results were also recorded for *Cannabis sativa* L. 'Finola' plants (Pagnani *et al.* 2018). The treatment with the consortium, improved plant growth, physiological status, and secondary metabolites.

The aim of the present study was to enhance the propagation of *A. umbelliformis* subsp. *eriantha* using synthetic seeds enriched with the rhizobacteria consortium. To evaluate the effectiveness of an improving agent, the treated synthetic seeds were compared to untreated synthetic seed, and followed through the *ex vitro* cultivations. To assess the differences between the obtained plants, the samples were investigated for their growth and development parameters, polyphenolic compounds, volatile fractions, antioxidant properties, and total phenolic and flavonoid content.

Materials and methods

All standards and reagents were purchased from Sigma-Aldrich® (St. Louis, MO). For High Performance Liquid Chromatography-diode array detection (HPLC-DAD) analysis, standard and reagents were HPLC-grade. For the other analyses, reagent-grade quality reagents were used. The experimental phases of the study are reported in Fig. 1.

Experimental design and data collection Two homologous experiments were carried out simultaneously. The *in vitro* experiments were completed in the micropropagation laboratory, while the *ex vitro* acclimatization and cultivations were performed in the greenhouse, both at the University of L'Aquila. The experiments were arranged in a completely randomized block design with three replicates, in which two different synthetic seed production strategies were compared: (i) enrichment with the PGPR consortium, and (ii) control without PGPR. For the *in vitro* experiment, a single experimental unit consisted of 150 synthetic seeds (three replicates of 50). For the *ex vitro* experiment, the single experimental unit consisted of 75 seedlings (three replicates of 25). At plant maturation, six plants per each replicate were sampled, and plant height (cm) was recorded. Healthy leaf apices were isolated from each plant and analyzed for their chlorophyll content (method described in Spectrophotometric assays section), and dry matter (samples were dried at 80°C until constant weight). Dry leaf apices, which belonged to the same experimental unit replicate, were homogenized and extracted for the different chemical analyses.

Plant growth-promoting rhizobacteria consortium *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Burkholderia ambifaria*, and *Herbaspirillum seropedicae* cultivation and PGPR consortium preparation, were obtained following the procedure previously described by Pagnani *et al.* (2018). After a matrix effect evaluation, the initial inoculum concentration was adjusted spectrophotometrically to 10^9 cells mL^{-1}, to obtain a final density of 10^6 cells g^{-1} with a ThermoFisher Scientific® Multiskan™ Go spectrophotometer (Thermo Fisher Scientific®, Waltham, MA).

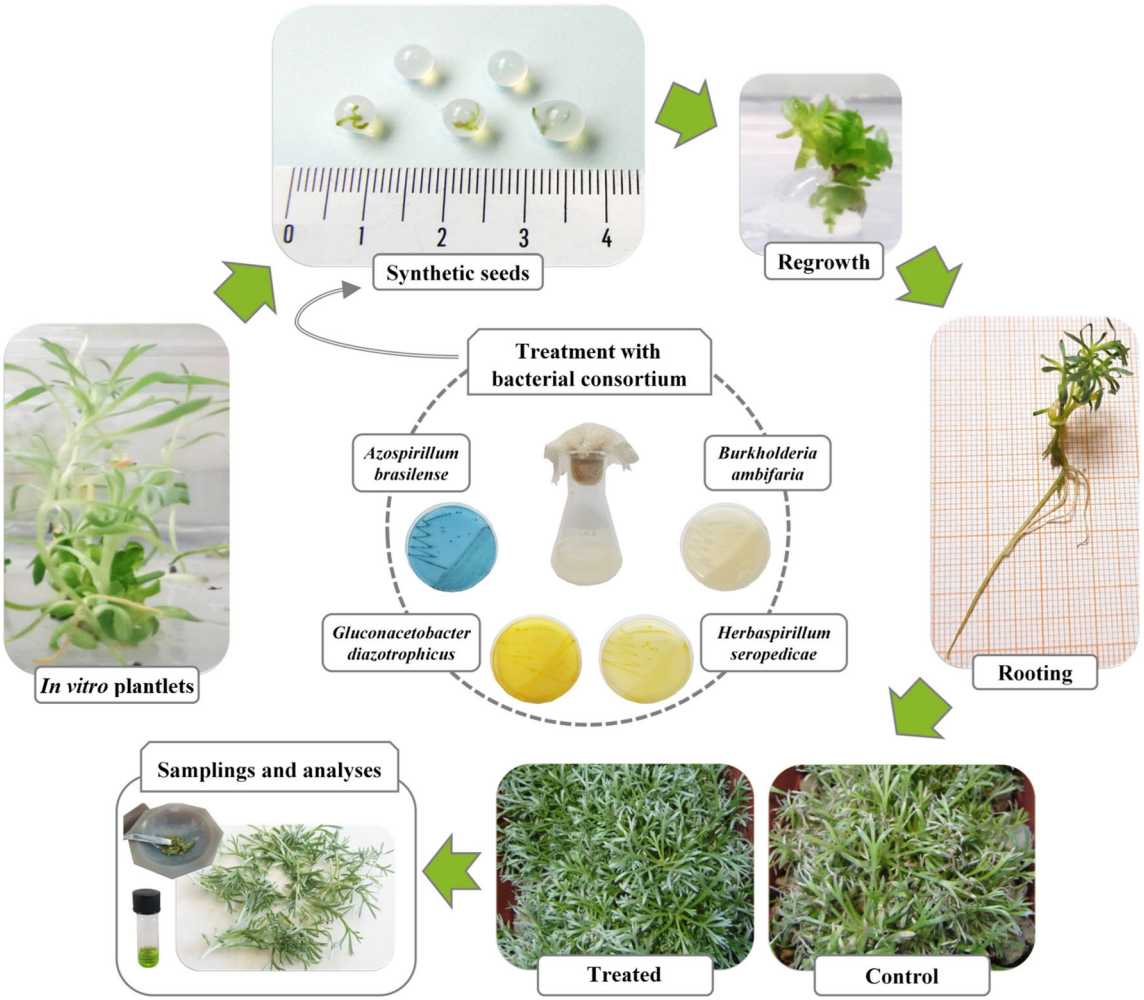

Fig. 1 Experimental phases of the study. Natural seeds of *Artemisia umbelliformis* Lam. subsp. *eriantha* were cultivated *in vitro* and microcuttings were encapsulated in synthetic seeds with and without bacterial enrichment. After *in vitro* regrowth, the plantlets were acclimatized for the *ex vitro* cultivation and followed until maturation. Plants were then studied for their plant growth and development parameters

Synthetic seeds preparation, *in vitro* germination, and *ex vitro* acclimatization *In vitro* plantlets were obtained from seeds collected on Monte Portella 2500 m asl (*Gran Sasso-Monti della Laga* National Park) in August 2016. Seed surface sterilization and *in vitro* germination were obtained as previously described in Pace *et al.* (2004). For control synthetic seed preparation, plantlet microcuttings (nodal segments of 3–4 mm length), were encapsulated with 2.5% (*w/v*) sodium alginate solution prepared in MS medium (Murashige & Skoog 1962), adjusted to pH 5.8 with 1 N HCl using a pH 8+ DHS BASIC pH meter (XS Instruments, Carpi, Italy), with BAP (6-Benzylaminopurine, 0.4 mg L^{-1}), and 3% sucrose (*w/v*), which was complexed for 25 min in a 50 mM $CaCl_2$ solution, and washed three times in sterile water. For synthetic seeds enriched by the PGPR consortium, the encapsulation agent was included the inoculum. *In vitro* germination percentages were evaluated on MS medium (pH 5.8), with 3% sucrose (*w/v*) and 0.7% plant cell culture tested agar (*w/v*). The germination curves of the treated and control synthetic seeds were prepared representing germination percentages (number of seeds germinated over the total number of planted seeds × 100) over time (9 wk). Once germinated, the shoots from control synthetic seeds were transferred to MS, adjusted to pH 5.8 with 1 N HCl, using pH 8+ DHS BASIC pH meter (XS Instruments, Carpi, Italy, with 0.5% (*w/v*) indole-3-butyric acid (IBA), 3% sucrose (*w/v*), 0.05% $CaCO_3$ (*w/v*), and 0.7% plant cell culture tested agar (*w/v*). The shoots obtained from the inoculated seeds were transferred to the same medium without IBA, to test the ability of the bacteria to induce rooting. The culture chamber was maintained at 26°C with a 12-h photoperiod, and an irradiance of 101 µmol m^{-2} s^{-1} provided by a cool white fluorescent lamp L 30 W 840 lumilux (Osram, Garching, Germany). The rooted plantlets were transferred to soil and kept in a greenhouse as previously described (Pace *et al.* 2010).

Spectrophotometric assays Spectrophotometric assays were completed using the Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific®, Waltham, MA). 100 mg of fresh leaf apices, isolated from sampled plants, were analyzed for chlorophyll content following the method and calculations described by Arnon (1949). Three replicates of dried homogenized matrix were sampled and extracted with two consecutive 30 min ultrasonic extractions, using 80% (*v/v*) methanol and 70% (*v/v*) acetone. The mixtures were centrifuged at 10,000 rpm for 5 min, and the supernatants were collected and pooled, evaporated, and resuspended with 1 mL of HPLC-grade methanol.

Extracts were analyzed for their antioxidant activities (AOC), by DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), and FRAP (Ferric reducing antioxidant power) assays. Ferric reducing antioxidant power (FRAP) was assessed by the potassium ferricyanide-ferric chloride method described by Oyaizu (1986). The DPPH assay was performed following the method proposed by Brand-Williams *et al.* (1995). The ABTS assay was carried out with the method proposed by Gullon *et al.* (2015). For all of the assays, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was used as the reference standard and results were expressed as mg Trolox Equivalents per g of dry weight. For DPPH and ABTS assays, different sample concentration solutions were also assayed to obtain the IC_{50} values (mg mL^{-1} concentration necessary to provide a scavenge radical activity of 50%).

Extracts were also evaluated for their total phenolic and flavonoid content (TPC and TFC, respectively). TPC estimation was carried out by Folin-Ciocalteu's reagent, following the method described by Singleton & Rossi (1965); gallic acid (GA) was used as a reference standard, and results were expressed as mg GA equivalents (GAE) per dry weight matrix. TFC was estimated by the Blasa *et al.* (2006) method using rutin as the reference standard, and results were expressed as mg rutin equivalents (RE) per g of dry weight.

HPLC-DAD polyphenolic profile analysis Sample extracts used for spectrophotometric assays were also characterized for their polyphenolic profiles by HPLC-DAD. Analyses were carried out with an HPLC system (Agilent 1200 series - Agilent Technologies - Santa Clara, CA), equipped with a DAD (Agilent 1200 series - Agilent Technologies). The separation module was equipped with a Kinetex C18 (5 μm, 100 Å, 30 × 3.0 mm, Phenomenex - Torrance, CA). The mobile phase was 1% (*v/v*) aqueous formic acid (A) and acetonitrile (B), with a flow rate of 0.4 mL min^{-1}. The separation of the selected analytes was obtained by a mobile phase gradient programmed as follows: B from 5% to 30% in 2 min; to 60% in 1 min; to 70% in 3 min; to 100% in 2 min, then switched back to the initial 5% in 1 min. The list of investigated analytes and their respective wavelengths are shown in Table S1 (Supplementary material). The quantitative analysis was performed by an Agilent ChemStation software (Agilent Technologies), with the calibration curve method (0.1–100 mg L^{-1}), and the results were expressed as g 100 g^{-1} dry weight.

GC-MS analysis Three replicates of the homogenized matrix were sampled and extracted with hexane in an ultrasonic bath for 30 min. The ultrasonicated samples were centrifuged at 5000 rpm for 5 min, and supernatants were recollected and stored at −40°C until analyzed. GC-MS analyses were carried out by a GC Clarus 580 (PerkinElmer - Waltham, MA), equipped with a fused silica Zebron™ ZB-SemiVolatile column (30 m × 0.25 mm 0.25 μm, Phenomenex), and coupled to Clarus GC/MS SQ (PerkinElmer). The extracts (1 μL) were directly injected into the column, and the separation of the analytes was obtained with a program ramp set as follows: from 50°C (holding 1 min), ramped at a rate of 5°C/min to 145°C (holding 15 min), ramped at a rate of 7°C/min to 175°C, and then ramped at a rate of 4°C/min to 250°C (holding 15 min). The carrier gas was helium at a flow rate of 1 mL/min; the injector temperature and the transfer line temperature were set at 250°C; the MS acquisition was in full scan mode (50 to 600 amu). Semi-quantitative analysis was obtained using Turbomass 6.1.0.1963 software (PerkinElmer), while the identifications of the volatile compounds were obtained by matching the mass spectra with the NIST Mass Spectral Library 2.0 (NIST - Gaithersburg, MD). The identity was confirmed by retention index calculated with a C4-C40 n-alkane series, as described by Lee *et al.* (1979), and compared with those present in the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/).

Statistical analysis One-way analysis of variance (ANOVA) was applied to test (*F-test*) the effects of treatment on all of the investigated variables. Means separation was performed by the Least Significant Difference test (LSD), at 0.1%, 1%, and 5% levels of significance. One-way ANOVA analyses and correlation tests (Pearson's), were performed using the XLSTAT 2016 software (Addinsoft, Paris, France).

Results

Synthetic seed germination, rooting, and *ex vitro* acclimatization The germination curves of the treated (PGPR) and control (CNT) synthetic seeds are represented in Fig. 2. The synthetic seed germination on MS medium occurred in both experiments after 3 wk. A significant difference ($p < 0.01$) in regrowth was evident from the beginning of the germination, with a higher percentage among the PGPR treatment (81%), compared to the control (63%). After 6 wk., no further germination was observed in either experiment.

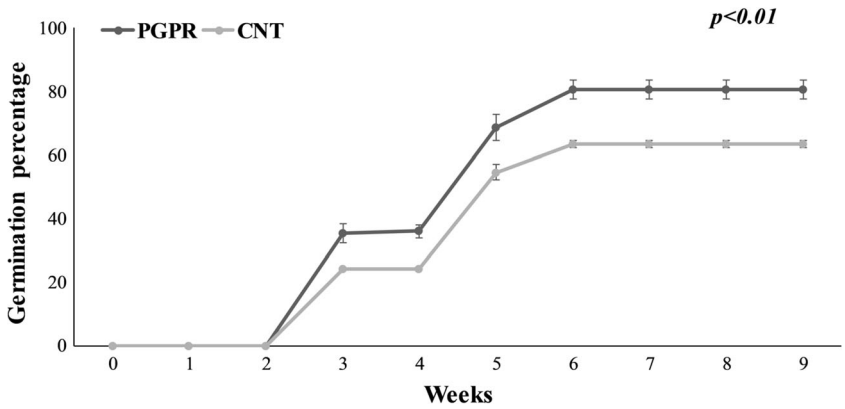

Fig. 2 Synthetic seed germination of Genepì with bacteria consortium (PGPR), and without treatment (CNT) over 9 wk.

The presence of PGPR also influenced the rooting stage (Table 1). The presence of the bacteria allowed the seedlings to root without the presence of IBA in the growth medium. The PGPR rooting activity obtained a performance equivalent to the IBA synthetic plant hormone. The recorded rooting percentage for enriched synthetic seeds (PGPR), showed no significant difference ($p > 0.05$) with the untreated. The average rooting percentage was 95%, and occurred in both media 3 wk. after shoot transfer.

The bacterial growth-promotion was crucial for the acclimation. The transfer from *in vitro* to *ex vitro* conditions, produced a different response among plantlets obtained from inoculated and control synthetic seeds (Table 1). The plant survival percentage was higher ($p < 0.01$), in plants obtained from inoculated synthetic seeds compared to the control. Furthermore, plants enriched with bacteria developed improved morphological and growth characteristics during *ex vitro* growth; they produced more chlorophyll ($p < 0.001$), had increased height ($p < 0.001$), and dry matter ($p < 0.001$).

Antioxidant activity and total phenolic and flavonoid content Results recorded for the FRAP assay showed that the highest ($p < 0.01$) antioxidant capacity was recorded for Genepì plants obtained from synthetic seeds enriched with PGPR (Table 2). The same results were recorded in ABTS and DPPH scavenging assays, and the IC_{50} values were lowest ($p < 0.001$) in samples obtained from synthetic seeds inoculated with the bacterial consortium (Table 2). The results from TFC and TPC assays are shown in Table 2. These spectrophotometric assays showed that the highest accumulation of flavonoids ($p < 0.001$) and phenolic compounds in general ($p < 0.01$), occurred in Genepì plants obtained from synthetic seeds enriched with PGPR. The Pearson correlation test carried out on AOC and TPC and TFC results, determined that there was a significant strong association among assessments. TPC values were positively associated with FRAP ($r = 0.857$; $p = 0.029$; $R^2 = 0.735$), and negatively with ABTS ($r = -0.634$; $p = 0.018$; $R^2 = 0.402$) and DPPH results ($r = -0.799$; $p = 0.056$; $R^2 = 0.638$). The opposite results were observed for TFC values, which correlated negatively with FRAP ($r = -0.924$; $p = 0.008$; $R^2 = 0.854$), and positively with ABTS ($r = 0.964$; $p = 0.002$; $R^2 = 0.930$) and DPPH ($r = 0.988$; $p = 0.001$; $R^2 = 0.976$).

Polyphenolic profiles The polyphenolic compounds detected in the samples and their quantification results are shown in Table 3. From the eighteen polyphenolic compounds evaluated (Table S1 Supplementary material), only fifteen were detected. The same qualitative profile was obtained in both experiments. The results of the quantitative analysis, on the other hand, identified some differences; except for *p*-coumaric acid ($p > 0.05$), significant differences in polyphenolic compound

Table 1 Different responses among *Artemisia umbelliformis* Lam. subsp. *eriantha* plantlets obtained from inoculated (PGPR) and control (CNT) synthetic seeds, recorded during rooting and acclimatization (survival) stages, and from plants after *ex vitro* growth (plant height, chlorophyll A, B, and total, and leaf dry matter)

Treatment	Rooting (%)	Survival (%)	Plant height (cm)	Chlorophyll A (mg g^{-1})	Chlorophyll B (mg g^{-1})	Total Chlorophyll (mg g^{-1})	Leaf Dry Matter (g 100 g^{-1})
CNT	94.72	65.33	6.64	0.22	0.20	0.43	13.84
PGPR	95.05	81.33	12.94	0.39	0.31	0.70	15.08
LSD	4.96	5.24	0.48	0.02	0.01	0.02	0.212
F-test	NS	**	*	*	*	*	*

CNT – Genepì without treatment; PGPR – Genepì inoculated by plantgrowth-promoting rhizobacteria. LSD, least significant difference between two means at $p < 0.001$ (*) and $p < 0.01$ (**) levels (Fisher's LSD test); NS, not significant

Table 2 Results obtained from total phenolic (TPC) and flavonoid content (TFC) methods, and ABTS, FRAP and DPPH antioxidant activity assays, carried out on extracts of dried leaf apices of inoculated (PGPR) and control (CNT) samples from *Artemisia umbelliformis* Lam. subsp. *eriantha*

Treatment	FRAP (mg TE g DW^{-1})	ABTS (IC_{50})	DPPH (IC_{50})	TFC (mg RE g DW^{-1})	TPC (mg GAE g DW^{-1})
CNT	9.77	32.18	37.60	24.15	2.23
PGPR	14.88	28.17	29.39	30.93	2.62
LSD	1.98	2.24	0.70	1.76	0.38
F-test	**	**	*	*	***

CNT – Genepì without treatment; PGPR – Genepì inoculated by plant-growth promoting rhizobacteria. LSD, least significant difference between two means at $p < 0.001$ (*), $p < 0.01$ (**) and $p < 0.05$ (**) levels (Fisher's LSD test)

concentrations between the experiments were been shown. Genepì plants obtained from untreated synthetic seeds, had the highest concentrations of 4-OH benzoic acid ($p < 0.001$), vanillic acid ($p < 0.05$), and ferulic acid ($p < 0.001$). However, the accumulation of the other eleven investigated polyphenolic compounds was higher ($p < 0.001$; rutin, $p < 0.01$) in Genepì plants obtained from synthetic seeds inoculated with PGPR compared to the controls.

Volatile fractions To evaluate the influence of PGPR on secondary metabolism, plant volatile fractions obtained from the different experiments were also compared. The qualitative and semi-quantitative characterization results obtained by GC-MS analysis, are shown in Table 4. The qualitative characterization revealed that volatile fractions in both Genepì experiments had the same chemical profile. In both experiments, the major compound was α-thujone, followed by β-thujone, and the other compounds contributed to the total abundance in percentages lower than 5%. The total level of identified compounds was of 92% (LSD 1.54). Alternatively, the semi-

Table 3 Polyphenolic profile concentrations from *Artemisia umbelliformis* Lam. subsp. *eriantha* obtained for dried leaf apices extracts of inoculated (PGPR) and control (CNT) samples, determined by means of HPLC-DAD analysis

Compound	CNT (μg g^{-1} DW)	PGPR (μg g^{-1} DW)	**LSD**	***F-test***
4-OH Benzoic acid	78.9	46.4	3.8	*
Catechin	52.9	114.5	6.9	*
Chlorogenic acid	404.6	631.4	16.2	*
Caffeic acid	24.7	46.9	3.0	*
Vanillic acid	87.6	77.5	7.7	***
Syringic acid	19.6	40.6	2.8	*
Rutin	436.3	541.1	63.9	**
p-Coumaric acid	170.8	172.5	19.5	NS
Ferulic acid	262.7	185.0	7.7	*
Sinapic acid	213.2	802.3	13.1	*
Cinnamic acid	10.9	18.1	1.7	*
Rosmarinic acid	161.8	269.5	5.3	*
o-Coumaric acid	267.4	420.6	16.9	*
Quercetin	79.9	113.2	9.2	*
Kaempferol	281.8	471.8	51.7	*
Total	2550.1	3951.3	114.7	*

CNT – Genepì without treatment; PGPR – Genepì inoculated by plant-growth promoting rhizobacteria. DW, dry weight NS, not significant; LSD, least significant difference between two means at $p < 0.001$ (*), $p < 0.01$ (**) and $p < 0.05$ (***) levels (Fisher's LSD test)

Table 4 Volatile fraction chemical composition of inoculated (PGPR) and control (CNT) dried leaf extracts, characterized by means of GC-MS analysis

Compound	RIE	RID	CNT (%)	PGPR (%)	LSD	*F-test*
α-Thujene	930	931	0.16	0.29	0.18	*
α-Pinene	936	939	1.41	0.91	0.20	**
β-Pinene	982	981	0.15	0.09	0.02	*
β-Myrcene	993	993	1.13	0.26	0.06	*
trans-β-Ocimene	1033	1032	1.03	0.62	0.11	*
p-Cymene	1038	1036	0.27	0.16	0.01	*
1,8-Cyneole	1050	1052	4.86	4.44	0.22	*
cis-Sabinene hydrate	1065	1063	1.56	0.90	0.06	*
γ-Terpinene	1089	1091	1.50	0.94	0.21	*
α-Thujone	1104	1105	51.49	55.89	0.64	*
β-Thujone	1119	1114	12.05	13.16	0.42	*
Myrtenal	1150	1151	1.02	0.67	0.06	*
Terpinen 4-olo	1175	1177	0.75	0.80	0.09	NS
α-Terpineol	1187	1189	1.42	1.29	0.35	NS
α-Copaene	1393	1392	0.95	0.60	0.15	**
Bergamotene	1436	1435	5.50	3.20	0.42	*
β-Caryophyllene	1421	1420	2.41	2.98	0.27	**
γ-Muurolene	1471	1473	0.11	0.07	0.02	**
Germacrene-D	1478	1480	3.43	4.54	0.74	**
Caryophyllene oxide	1580	1582	0.16	0.12	0.01	**

RIE, experimental retention index referred to C8–C40 n-alkane mixture standard; RID, retention index retrieved from http://webbook.nist.gov/chemistry/ for the same analysis conditions; CNT – Genepì without treatment; PGPR – Genepì inoculated by plantgrowth promoting rhizobacteria. NS, not significant; LSD, least significant difference between two means at $p < 0.001$ (*) and $p < 0.01$ (**) levels (Fisher's LSD test)

quantitative characterization, highlighted several differences between plants obtained from enriched and control synthetic seeds. Except for α-terpineol and terpinen 4-olo, between the two experiments, a different relative abundance of compounds was detected. The volatile fractions obtained from plants derived from enriched synthetic seeds, recorded the highest relative abundance of the major compounds α-thujone and β-thujone ($p < 0.001$). The volatile fractions of these plants also had the highest percentages of β-caryophyllene ($p < 0.01$) and germacrene-D ($p < 0.01$). The relative abundance levels of the other compounds, however, were higher ($p < 0.001$, and $p < 0.01$ for α-pinene, α-copaene, ϒ-muurolene, and caryophyllene oxide), in plants obtained from control synthetic seeds compared with plants obtained from the treated seeds.

Discussion

In the present study, the bacterial consortium formed by *Azospirillum brasilense, Gluconacetobacter diazotrophicus, Burkholderia ambifaria*, and *Herbaspirillum seropedicae*, was utilized for the first time as an enrichment agent of Apennines Genepì (*Artemisia umbelliformis subsp. eriantha*) synthetic seeds. The ability of the consortium to promote plant growth and development, was apparent during the different stages of plant growth. During the *in vitro* germination and rooting stages, and *ex vitro* acclimatization and growth, the presence of PGPR improved most of the parameters investigated.

The presence of PGPR promoted the germination of a higher number of synthetic seeds of a high-altitude plant species. It was already determined that germination improved for other plants at lower altitudes. For example, Almaghrabi *et al.* (2014) reported that the treatment of *Zea mays* L. with strains belonging to *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Serratia marcences*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus amyloliquefaciencs*, increased seed germination by 7 to 13% compared to the control. The same results were reported in the *in vivo* study of Gholami *et al.* (2009), in which the PGPR consortium of six strains belonging to *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Azospirillum brasilense* species, increased the *Zea mays* seed germination to 18.5% compared to the untreated control. In addition, Marathe *et al.* (2017), reported the highest *Glycine max* L. germination rates in the presence of a *Pseudomonas aeruginosa* strain inoculation.

Shoots of Genepì with PGPR were able to convert into seedlings without the addition of IBA in the MS medium. Similar findings were reported by Larraburu and Llorente (2015). These researchers found that the addition of a strain of *Azospirillum brasilense,* reduced the IBA concentration required (by 96%), for the optimal *in vitro* rooting of *Handroanthus impetiginosus* (Mart. Ex. DC) Mattos, on half-strength woody plant medium. Russo *et al.* (2008) reported that even if the presence of a strain of *Azospirillum brasilense* improved the *Prunus cerasifera* L. clones root systems, the addition of IBA was indispensable to promote explant rooting. These germination and rooting abilities can be ascribed to a PGPR direct growth promotion by: i) beneficial compound production (hormones, enzymes, *etc.*), ii) facilitating nutrient uptake, iii) and siderophore synthesis and secretion (Glick 1995). The acclimatization step also showed the value of PGPR enrichment in synthetic seeds. These results are in accordance with those reported by Larraburu *et al.* (2007), who recorded high acclimatization results and increased *ex vitro* survival in inoculated *Photinia serrulata* Lindl. plants (75–100% for inoculated plants compared to 50–60% for control plants). Similar findings were also reported by Suada *et al.* (2015), who reported that inoculations with *Pseudomonas putida*, *Pseudomonas flourescens*, and particularly *Bacillus* sp. had positive effects on acclimatization and hardening of micropropagated *Musa acuminata* cv. Grand Naine.

The PGPR contribution during *in vitro* and *ex vitro* steps produced plants that thrived the best. The experimental units of PGPR, recorded the tallest plants with the most chlorophyll and dry matter. These findings are in accordance with the already recognized plant growth and development promotion associated with PGPR groups (Dar *et al.* 2018), and to the investigated consortium (Botta *et al.* 2013; Pagnani *et al.* 2018).

The positive effects of the enrichment of synthetic seeds with PGPR consortium, were seen not only in plant growth and development parameters, but also in the spectrophotometric assays and the HPLC-DAD analysis results. In general, the highest accumulation of secondary metabolites was observed in plants obtained from enriched synthetic seeds. The antioxidant capability was statistically correlated primarily to phenolic compounds, as usually reported for different plant species (Piluzza and Bullitta 2011). Regarding GC-MS characterization, PGPR only seemed to induce the accumulation of the major compounds of the volatile fractions. Nevertheless, the profile was similar to those reported previously (Pace *et al.* 2010; Fasciani *et al.* 2017). The current findings are in accordance with literature data, and it has been documented by several researchers that the use of the PGPR induces plant secondary metabolism and antioxidant activity in other plant species (Jain and Patriquin, 1985; Kilam *et al.* 2015; Kiprovski *et al.* 2016; Khalid *et al.* 2017; Pagnani *et al.* 2018).

Conclusions

High altitude plants are affected by habitat modifications due to climate change (Stanisci *et al.* 2005). In addition, plants

with medicinal properties, such as Apennines' Genepì, are also threatened by undiscerning collections (Pace *et al.* 2010). Human intervention is important to avoid extinction, as plants can be translocated to achieve conservation (Reiter *et al.* 2018), and bacterial implementation can be utilized to optimize plant propagation (Sahoo *et al.* 2012). The results of this study show that the investigated PGPR consortium is a good candidate as an enrichment agent in synthetic seed technology, because it improved plant propagation performance and development. These findings represent a valid basis for more extensive evaluation of the propagation of plants that belong to endangered and/or endemic species.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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