OPEN ACCESS

RESEARCH ARTICLE

Spanish Journal of Agricultural Research 22 (4)
October-December, 2024, 21014
ISSN-L: 1695-971X, eISSN: 2171-9292
https://doi.org/10.5424/sjar/2024224-21014

Ex vitro propagation and phytochemical analysis of *Serapias vomeracea* (Burm.f.) Briq.: contribution to the conservation of Orchidaceae species

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Abstract

Aim of study: The primary objective of this study was to investigate the seed germination and antioxidant properties of *Serapias vomeracea* (Burm.f.) Briq. Specifically, the aims of the research were to explore the influence of the *Tulasnellaceae* spp, fungal isolate SVL-30 (MK250656), on germination and seedling development, compare antioxidant activity to that of ascorbic acid, analyse the phytochemical composition, and identify bioactive compounds present in the methanol extract.

Area of study: University of Ondokuz Mayıs, Faculty of Sciences, Department of Biology, Samsun, Türkiye

Materials and methods: Pots containing *S. vomeracea* and SVL-30 fungus, along with a control group without fungus, were utilized for the experiment. The impact of the fungus on germination stages and seedling development was assessed. Antioxidant analysis involved determining phenolic and flavonoid content, as well as DPPH radical scavenging activity (IC_{50}: 2.09 mg/mL). Chlorophyll and carotenoid contents were measured to evaluate the physiological health of the plant. GC-MS analysis was employed to identify 19 bioactive compounds present in the methanol extract.

Main results: The fungus significantly stimulated germination, with 83.02% of seeds germinating, and 52.66% progressing to the seedling stage. Antioxidant analysis revealed substantial phenolic and flavonoid content in *S. vomeracea* seedlings, demonstrating potent antioxidant properties comparable to ascorbic acid. Chlorophyll and carotenoid contents emphasized the balanced and healthy physiology of the plant. GC-MS analysis identified 19 bioactive compounds in the methanol extract, highlighting the potential bioactivity of *S. vomeracea*.

Research highlights: This study furnishes valuable information on the germination, phytochemical composition, and antioxidant capacity of *S. vomeracea* seedlings. The research underscores the potential bioactivity of the plant, substantiated by the identification of bioactive compounds. The findings lay the groundwork for further exploration of the potential health benefits of *S. vomeracea*. A strategic shift towards studies emphasizing sustainable agricultural practices is recommended, aiming to balance both conservation and utilization objectives.

keywords: Antioxidant properties; GC-MS; Seed germination; Sustainable agriculture practices.

Propagación ex vitro y análisis fitoquímico de *Serapias vomeracea* (Burm.f.) Briq.: contribución a la conservación de especies de Orchidaceae

Resumen

Objetivo del estudio: El principal objetivo de este estudio fue investigar la germinación de semillas y las propiedades antioxidantes de *Serapias vomeracea* (Burm.f.) Briq. Específicamente, la investigación buscó explorar la influencia de *Tulasnellaceae* spp., aislado SVL-30 (MK250656) en la germinación y el desarrollo de plántulas, comparar la actividad antioxidante con la del ácido ascórbico, analizar la composición fitoquímica e identificar los compuestos bioactivos presentes en el extracto de metanol.

Área de estudio: Universidad de Ondokuz Mayıs, Facultad de Ciencias, Departamento de Biología, Samsun, Turquía.

Materiales y métodos: Se utilizaron macetas que contenían *S. vomeracea* y el hongo SVL-30, junto con un grupo de control sin hongo. Se evaluó el impacto del hongo en las etapas de germinación y el desarrollo de las plántulas. El análisis antioxidante incluyó la determinación del contenido de fenoles y flavonoides, así como la actividad de eliminación

de radicales DPPH (IC_{50}: 2,09 mg/mL). Se midieron los contenidos de clorofila y carotenoides para evaluar la salud fisiológica de la planta. Se empleó análisis GC-MS para identificar 19 compuestos bioactivos presentes en el extracto de metanol.

Principales resultados: El hongo estimuló significativamente la germinación, con un 83,02% de semillas germinadas y un 52,66% que progresaron al estado de plántulas. El análisis antioxidante reveló un contenido sustancial de fenoles y flavonoides en las plántulas de *S. vomeracea*, mostrando potentes propiedades antioxidantes comparables al ácido ascórbico. Los contenidos de clorofila y carotenoides resaltaron la fisiología equilibrada y saludable de la planta. El análisis GC-MS identificó 19 compuestos bioactivos en el extracto de metanol, destacando la potencial bioactividad de *S. vomeracea*.

Aspectos destacados de la investigación: Este estudio proporciona información valiosa sobre la germinación, la composición fitoquímica y la capacidad antioxidante de las plántulas de *S. vomeracea*. La investigación subraya la potencial bioactividad de la planta, respaldada por la identificación de compuestos bioactivos. Los hallazgos sientan las bases para una mayor exploración de los posibles beneficios para la salud de *S. vomeracea*. Se recomienda un cambio estratégico hacia estudios que enfaticen prácticas agrícolas sostenibles, con el objetivo de equilibrar tanto la conservación como los objetivos de utilización.

Palabras clave: Propiedades antioxidantes; GC-MS; Germinación de semillas; Prácticas agrícolas sostenibles.

The translation of the title, abstract, and keywords from the original version in English to Spanish has been generated using OpenAI, ChatGPT GPT-4o mini (2024)

La traducción al español del título, resumen y palabras clave de la versión original en inglés ha sido generada utilizando OpenAI., ChatGPT GPT-4o mini (2024)

Citation: Çolak, E; Aytar, EC; Özdener Kömpe, Y (2024). Ex vitro propagation and phytochemical analysis of *Serapias vomeracea* (Burm.f.) Briq.: contribution to the conservation of *Orchidaceae* species. Spanish Journal of Agricultural Research, Volume 22, Issue 4, 21014. https://doi.org/10.5424/sjar/2024224-21014

Received: 23/01/2024. **Accepted**: 16/06/2024. **Published**: 22/11/2024

Introduction

The Mediterranean region exhibits an extremely heterogeneous geographical and ecological structure (Thompson, 2005). Since the political boundaries of this region do not fully correspond to bioclimatic, biogeographical, or floristic outlines, it is considered a challenging area to characterize. The Mediterranean Basin encompasses approximately 24-25.000 plant species (or taxa, including subspecies) (Vereecken, 2010). Therefore, it is recognized as one of the world's most important hotspots in terms of biological diversity and ranks second globally in terms of plant endemism (Camilleri et al., 2024).

Among plant groups, orchids exhibit high biological diversity, with European-Mediterranean orchids comprising over 640 species belonging to 35 genera (Delforge, 2016). Orchids, especially those growing in the Eastern Mediterranean, constitute a significant portion of this rich biological diversity. However, due to threats such as habitat loss, climate change, and illegal collection, the conservation of these orchids is of paramount importance. Conservation efforts should aim to ensure the sustainable management of this unique plant group (Pellegrino et al., 2021).

We live in a dynamically changing world, where changes in biological diversity are constantly documented on broad and complex scales. Therefore, while many species are experiencing a global decline, the distributions of alternative species are expanding, posing significant threats to local biological diversity in certain cases (e.g., invasive, non-native species) (Tsiftsis & Kindlmann, 2023).

Similar challenges are encountered in Türkiye regarding the conservation and trade of orchids. Orchids found in the country also have small populations and limited habitats, thus facing threats such as over-collection and trade. Türkiye richness in biodiversity and hosting various orchid species further emphasizes the importance of their conservation and sustainable trade. Concerns exist regarding the presence of illegal activities in the trade of orchids in Türkiye (Aytar et al., 2024). In particular, the illegal collection and trade of rare or protected orchid species endanger their populations. However, legal trade and conservation efforts are also in place. It is important to note that Türkiye is a party to international agreements regarding the conservation and trade of orchids, such as Convention on the International Trade in Endangered Species of Wild Flora and Fauna (CITES) and adheres to regulations (Fay, 2018). Nevertheless, effective implementation and monitoring of legal regulations are necessary. Currently, the asymbiotic germination of orchid seeds is considered the simplest method for producing a large quantity of seedlings and is widely utilized in commercial production across numerous orchid species (Chen et al., 2015). However, symbiotic seed germination is regarded as advantageous for species restoration and is believed to be more suitable for enhancing their

adaptation to nature (Sánchez-Gutiérrez et al., 2023; Tokuhara et al., 2023). The presence of compatible symbiotic fungi in orchid seedlings provides a more robust adaptation to the environment, resulting in higher survival rates and faster growth compared to asymbiotic seedlings. Recent attention has been drawn to research conducted in the presence of compatible symbiotic fungi. Particularly, studies conducted on *Anacamptis sancta* (L.) R.M.Bateman, Pridgeon & M.W.Chase 1997 (Deniz et al., 2022), *Anacamptis coriphora* (L.) R.M.Bateman, Pridgeon & M.W.Chase 1997 (Aytar et al., 2023), *Anacamptis laxiflora* (L.) R.M.Bateman, Pridgeon & M.W.Chase 1997 (Aytar & Kömpe, 2023), and *Serapias orientalis* (Greuter) H.Baumann & Künkele 1988 (Aytar & Kömpe, 2024) have laid the groundwork for bringing these species from seed to the flowering stage, contributing to both conservation efforts and sustainable agriculture.

Serapias vomeracea exhibits a wide natural distribution across the Balkans, France, Greece, Italy, Morocco, Portugal, Spain, Tunisia, and Türkiye (Acemi, 2020). Its flower morphology and pollination system are distinctive. The flower is supported by a bract of similar color. The apical part of the lip, termed as the epichile, is downwardly directed. The basal part (hypochile) and two lateral lobes are arranged in a small, dark-colored tube along with the perigonium, with sizes varying among taxa (Baumann, 1989). The pollination system occurs through various insects, allowing for the transfer of pollen among different species (Dafni et al., 1981; Lanzino et al., 2023).

The aim of this study was to elucidate the phytochemical profiles of *S. vomeracea* seeds by examining their germination process and assessing the effect of the *Tulasnellaceae* spp., fungal isolate SVL-30 (MK250656) (Kömpe et al. 2022) on this process. Additionally, the aims of the study were to determine the antioxidant properties and analyse the phytochemical composition of the plant. Finally, by identifying bioactive compounds present in the methanol extract, the study aims to understand the potential health benefits and biological activity of *S. vomeracea* and provide data for the conservation of the species.

Material and methods

Plant materials

For this research, seeds of *S. vomeracea* naturally growing in Samsun, Türkiye, were procured from mature capsules. The collected mature capsules were air-dried at room temperature. Subsequently, the seeds were extracted in the laboratory and stored at 4°C until utilized in germination tests.

In the study by Kömpe et al. (2022), the following method was employed for fungal isolation from the roots of *S. vomeracea*: Prior to root sample collection, the phenological stages of *S. vomeracea* were monitored for one year within its habitat (Ondokuz Mayıs University Campus). It was observed that leaves developed in February and March, while the roots had completely dried by July. For fungal isolation, roots were collected from one plant every month during the years 2015-2016. Sections of the roots were examined under a microscope for the presence of fungi. Roots containing fungal coils were sterilized in a 1.5% NaOCl solution for 5 minutes and washed with sterile distilled water. Under aseptic conditions, root segments (1-2 cm) were placed into Petri dishes containing a fungal isolation medium (FIM) and incubated in the dark at 27°C for 2 days. After examination under a stereo microscope, fungal colonies were transferred to FIM and purified. The pure fungal cultures were stored at 4°C. This method proved to be effective for fungal isolation and the establishment of pure cultures.

In this study, the fungal isolate SVL-30 (MK250656) previously isolated and purified using the method described above, was utilized. To stimulate its growth, the SVL-30 was transferred to potato dextrose agar (PDA) medium and incubated in the dark at 25°C for five days.

Ex vitro symbiotic seed germination

In this study, inspired by the method of Rasmussen and Whigham (1993), we aimed to investigate mycorrhizal symbiosis by placing orchid seeds in trays and embedding previously isolated fungal strain as small agar blocks into the soil mixture. In this approach, the orchid seeds were arranged in a specific pattern in the trays, and each tray was vertically placed just below the soil surface. The seeds were maintained in the soil mixture, creating an environment where mycorrhizal fungi could potentially establish colonies. The previously isolated SVL-30 was prepared as small agar blocks and incorporated into the soil mixture. These blocks were strategically placed within the soil to initiate mycorrhizal colonization. The seeds within the trays were subjected to conditions where they could potentially form a symbiotic relationship with the mycorrhizal fungi during germination. This method facilitated the successful identification of mycorrhizal relationships and allowed us to examine the effects of the isolated SVL-30 on orchid seed germination.

After approximately 60 days, the germination and growth status of the seeds in the packets were evaluated. As a control group, seed packets were placed in sterilized soil without fungal inoculation. These packets underwent the same watering and monitoring procedures as the pots with fungal compost. Three pots were prepared for fungal isolate, and each pot contained three seed packets.

Developmental stages were assessed based on modified criteria established by Clements et al. (1984), as follows: 0 - Ungerminated seed, 1 - Protocorm, 2 First photosynthetic leaf, 3 - First leaf, 4 - Developed leaves and/or roots.

Phytochemical analysis of seedlings

Seeds were collected from pots and washed underwater to eliminate unwanted particles. The washed seedlings were then dried in an oven at 40°C. The dried samples were ground into a powder using a grinder (Aytar et al., 2020). The powdered samples were subjected to extraction according to the method by Aytar et al. (2024), where 0.5 g of dry sample was extracted with 10 mL of 80% methanol at 35°C for 24 hours. After cooling the samples to room temperature, they were centrifuged at 3500 x g for 10 min. The supernatant was then recovered for determining phytochemical contents (Cai et al., 2004).

Estimation of photosynthetic pigments

Photosynthetic leaf pigments, including chlorophyll a and b, carotenoids, lycopene, and beta-carotene contents, were determined using a spectrophotometric approach based on the method by Lichtenthaler (1987). Fresh seedlings weighing 0.5 g were placed in a small bottle containing 10 mL of 80% ethanol. Bowls for pigment extraction were wrapped in aluminium foil and kept in the dark for seven days. Using a spectrophotometer, absorbance (A) at wavelengths 663, 645, 645, 505, and 453 nm was measured to determine concentrations of chlorophyll, lycopene, beta-carotene, and carotenoids (Shimadzu UV-2550, Kyoto, Japan). The following formulas were employed to determine photosynthetic pigments:

Total Chlorophyll = Chlorophyll a + Chlorophyll b

Chlorophyll a = (0.999 × A 663 − 0.0989 × A 645)

Chlorophyll b = (−0.328 × A 663 + 1.77 × A 645)

Lycopene = (0.0458 × A 663 + 0.204 × A 645 + 0.372 × A 505 − 0.0806 × A 453)

Beta-carotene = (0.216 × A 663 − 1.22 × A 645 − 0.304 × A 505 + 0.452 × A 453)

Carotenoids = (A 480 + (0.114 × A 663 − 0.638 × A 645))

Total phenolic content determination

The total phenolic content was determined using the Folin–Ciocalteu method. Following appropriate dilution according to Singleton and Rossi (1965) method, 200 µL of plant sample was added to 1 mL of 0.2 N Folin–Ciocalteu reagent. After 4 min, 800 µL of saturated Na_2CO_3 solution (approximately 75 g/L) was added. After incubation at room temperature (20°C) for 2 hours, absorbance was measured at 760 nm (Singleton and Rossi 1965). Gallic acid was used to calibrate the standard curve. The total phenolic content was expressed as milligrams of gallic acid equivalent (mg GAE)/g of plant material's dry weight.

Determination of total flavonoid content

Total flavonoid content was determined using the aluminium chloride colorimetric analysis. According to Mahmud et al. (2017), in 1 mL extract solution (1 mg/mL), 4 mL distilled water and 0.3 mL sodium nitrate ($NaNO_3$) (50 g/L) were sequentially mixed. After 5 min, 0.3 mL aluminium chloride ($AlCl_3$) (100 g/L) was added with continuous shaking. At the sixth minute, 2 mL of 1 M sodium hydroxide (NaOH) was added, and after adjusting the volume to 10 mL, absorbance was measured at 510 nm. Quercetin was used to prepare the standard calibration curve, and the total flavonoid content of the extract was expressed as milligrams of quercetin equivalent (QE)/g of dry extract.

Determination of total flavonol content

Total flavonol content was determined by a colorimetric analysis. According to Grubešić et al. (2005), 1 mL of extract and/or rutin working solution (standard) were mixed with 1 mL of 20 mg/mL $AlCl_3$ and 3 mL of 50 mg/mL sodium acetate (NaOAc) working solution, and the mixture was left at room temperature for 2.5 h. The absorbance of the mixture was measured at 440 nm. The value of total flavonol content was expressed as milligrams of quercetin equivalent (mg QE/g) of dry extract.

Determination of total proanthocyanidin content

Total proanthocyanidin content was determined by a colorimetric assay. According to Oki et al. (2002), extracts were mixed with appropriate dilution ratios of 2.5 mL vanillin working solution (30 mg/mL) and 2.5 mL sulfuric acid-methanol (30%) working solution. After incubating the mixtures for 20 min at 30°C, absorbance was measured at 500 nm. The value of proanthocyanidin content was expressed as milligrams of epicatechin equivalent (mg ECE/g) of dry extract.

Determination of total condensed tannin content

The determination of total condensed tannin content was carried out using a colorimetric method. According to Price et al. (1978), 50 µL of the extract was mixed with 3 mL of 4% vanillin (vanillin: MeOH, v/v) and 1.5 mL HCl. After 15 min of incubation at room temperature, absorbance was measured at 500 nm. The value of condensed tannin content was expressed as milligrams of catechin equivalent (mg CE/g) of dry extract.

Phytochemical profile of seedlings

The GC-MS analysis was conducted using a SHIMADZU GCMS-QP2010 Mass Spectrometer and AOC-5000 Auto Injector. A scanning range of 30-450 Da was employed on an Rxi-5MS column (30m × 0.25mm × 0.25 µm). The electron ionization system operated at 70 eV ionization energy, utilizing helium gas with a constant flow rate of 1 mL/min. The injection volume was set at 1.5 µL with a split ratio of 10:1, while the injector temperature and ion source temperature were maintained at 250°C and 200°C, respectively. The oven temperature was initiated at 70°C for 10 min, followed by a gradual increase of 3°C/min to 150°C, holding for 5 min. Subsequently, the oven temperature was raised by 10°C/min to 250°C, where it was held for 5 min. The solvent delay ranged from 0 to 2 min, resulting in a total GC/MS run time of 56.67 min. For liquid sampling, orchid samples extracted with methanol were diluted 100 times and placed in 1.5 mL vials. Analysis was conducted using the NIST Standard Reference database (Aytar et al., 2024).

Statistical analyses

To determine the relationship between two variables, correlation coefficients (R) were calculated using the CORREL statistical function in MS Excel software. Data are expressed as mean ± SD obtained from three separate observations. Especially, to ensure the accuracy of these calculated standard deviations and the consistency of the results, they were rechecked using SPSS 21 software.

Results

Seed germination of *S. vomeracea*

The *ex vitro* germination developmental stages of orchid seeds in pots with *S. vomeracea* and SVL-30 and in pots without fungus (control) are shown in Table 1. The SVL-30 used in germination stimulated both germination and seedling development. Protocorm formation occurred 25 days after sowing, and the germination and development status in the packets were recorded after 45 days. According to these results, it was determined that 83.02% (±4.93) of the seeds germinated, and they progressed to the seedling

Table 1. Ex vitro symbiotic seed germination and seedling development of *Serapias vomeracea*.

	Developmental stage %					
	S0	**S1**	**S2**	**S3**	**S4**	**Total Germination %**
S. vomeracea+SVL-30	13.50±4.50	7.15±2.83	15.71±2.08	10.94±4.36	52.66±5.40	83.02±4.93
S. vomeracea (Control)	100±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

The results are expressed as mean ± standard deviation, n = 3.
S : Stage
SVL-30: *Tulasnellaceae spp*.(MK250656) Kömpe et al., 2022)

Table 2. Antioxidant properties of *Serapias vomeracea* seedlings.

Material	**DPPH ($IC_{50}\mu$ g/mL)**	**Total flavanol content (mg QE/g extract)**	**Total flavonoid content (mg QE/g extract)**	**Total phenolic content (mg GAE/g extract)**	**Total proanthocyanidin content (mg CAE/g extract)**	**Total tannin content (mg GAE/g extract)**
S. vomeracea of seedling	2.09±0.02	41.83±25.57	245.20±54.01	131.45±2.29	144.31±23.34	3.37±0.01

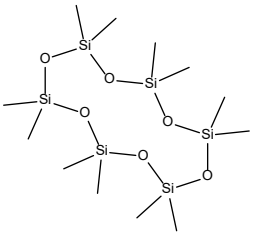
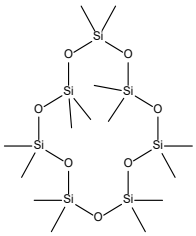
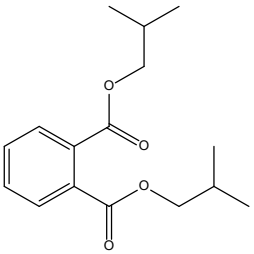
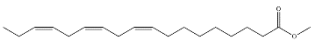
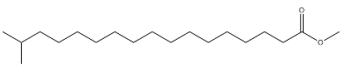
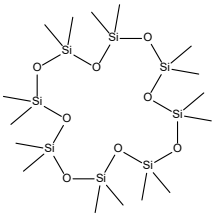
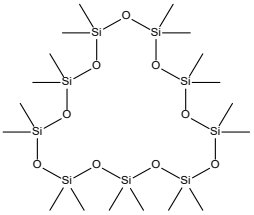
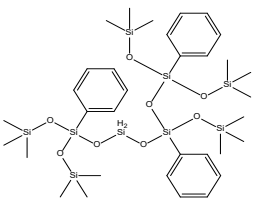
The results are expressed as mean ± standard deviation, n = 3.

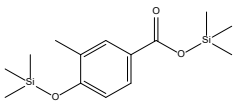
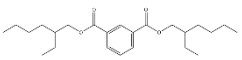
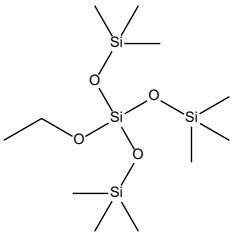
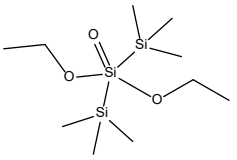
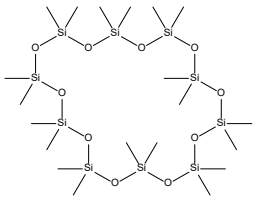
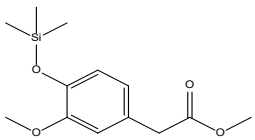
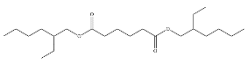
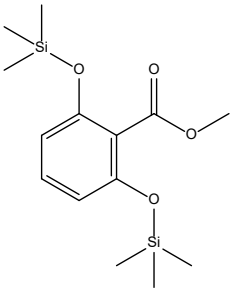
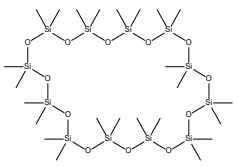
Table 3. Amounts of chlorophyll a and b, beta-carotene, and lycopene in seedlings.

Material	**Chlorophyll a**	**Chlorophyll b**	**Total chlorophyll**	**Beta-carotene**	**Lycopene**
S. vomeracea of seedling	9.33±0.14	17.08±0.48	26.41±0.47	0.37±0.05	0.401±0.007

The results are expressed as mean ± standard deviation, n = 3.

Table 4. GC-MS results of methanol extract of *Serapias vomeracea* seedlings.

No	RT (min)	Name of the compound	Structure	Area %
1	24.433	Cyclohexasiloxane, dodecamethyl-		1.80
2	32.287	Cycloheptasiloxane, tetradecamethyl-		1.04
3	46.197	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester		1.70
4	49.786	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-		1.00
5	50.101	Heptadecanoic acid, 16-methyl-, methyl ester		0.65
6	50.281	Cyclooctasiloxane, hexadecamethyl-		0.71
7	50.595	Cyclononasiloxane, octadecamethyl-		10.51
8	50.779	Bis[di(trimethylsiloxy)phenylsiloxy] trimethylsiloxyphenylsiloxane		0.60

9	51.037	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy] benzoate		2.64
10	51.139	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester		0.87
11	51.935	Tetracosane		2.27
12	52.133	3-Ethoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsilyloxy) trisiloxane		1.83
13	52.302	Silicic acid, diethyl bis(trimethylsilyl) ester		4.07
14	52.579	Cyclodecasiloxane, eicosamethyl-		3.48
15	52.733	Benzeneacetic acid, 3-methoxy-4-[(trimethylsilyl)oxy]-, methyl ester		3.07
16	53.065	Hexanedioic acid, bis(2-ethylhexyl) ester		4.97
17	53.683	Methyl 6,6,8,8,10,10-hexamethyl-3-oxo-2,5,7,9,11		2.90
18	54.480	Benzoic acid, 2,6-bis(trimethylsiloxy)-, methyl ester		2.80
19	55.773	Tetracosamethyl-cyclododecasiloxane		28.83

stage (Stage 4) with developing leaves at a rate of 52.66% (±5.40). No germination occurred in the seeds without fungus. The developmental stages (S2, S3 and S4) are illustrated in Figure 1.

Antioxidant and phytochemical analysis results

he antioxidant properties of *S. vomeracea* seedlings were evaluated, showing an IC_{50} value of 2.09 µg/mL for DPPH activity, with notable levels of total flavanol, flavonoid, phenolic, proanthocyanidin, and tannin contents Table 2. Furthermore, in Table 3, the seedlings exhibited significant amounts of chlorophyll a, chlorophyll b, total chlorophyll, beta-carotene, and lycopene. According to the results obtained, the chlorophyll content of *S. vomeracea* is quite balanced and healthy.

Detection of bioactive compounds from GC-MS

We identified various bioactive contents from the methanol extract of seedling of S. vomarecea, a terrestrial orchid. The retention time (RT), molecular formula, molecular weight (MW), and concentration (% area) of 19 bioactive phytochemical compounds in the methanol extract are shown in Table 4. GC-MS analysis of the methanol extract from *S. vomeracea* seedlings, highlighting several major compounds. The most abundant compounds include Tetracosamethyl-cyclododecasiloxane (28.83%), Cyclononasiloxane, octadecamethyl- (10.51%), and Hexanedioic acid, bis(2-ethylhexyl) ester (4.97%). Other notable compounds include Silicic acid, diethyl bis(trimethylsilyl) ester (4.07%) and Cyclodecasiloxane, eicosamethyl- (3.48%). These siloxane-based compounds are predominantly present in the extract, potentially contributing to its unique properties.

Discussion

The successful germination, conservation, propagation, and cultivation of numerous orchid species are fundamental prerequisites for the establishment of *in vitro* orchid seedling production programs (Kaladharan et al., 2024). Nevertheless, the nutritional requirements for *in vitro* germination exhibit substantial variability not only among different species but also within taxa originating from diverse habitats within the same geographical regions, potentially reflecting locally adapted ecotypes. It is noteworthy that the chemical inputs and energy expenditures associated with *in vitro* production tend to be comparatively higher (Shao et al., 2024). Conversely, *ex vitro* seedlings offer a distinctive advantage by thriving in both energy-efficient and aseptic conditions, accompanied by the absence of chemical utilization (Aytar & Kömpe 2024). Previous research endeavors employing the *ex vitro* symbiotic method have yielded commendable results. Various studies have demonstrated that obtaining *ex vitro* seedlings that form symbiotic relationships with compatible fungi, their transfer to nature, and their survival are easier and more successful in the restoration of orchids (Aewsakul et al., 2013; Kömpe & Mutlu, 2021; Kömpe et al., 2022). Orchid seeds cannot germinate and develop into mature individuals without establishing a symbiotic relationship with certain fungi in nature; therefore, it is only possible to form a population depending on the abundance of fungi in the soil (McCormick et al.,2018).

In a previous study conducted in Türkiye by Deniz et al. (2022), *Anacamptis sancta* was propagated from seed and reintroduced into its natural environment. The fungus isolated from *A. sancta* roots was molecularly identified and found to belong to the Ceratobasidiaceae (ON939729). To determine the most compatible fungus for seed germination and seedling development, *A. sancta* seeds were inoculated under *ex vitro* conditions in sterile soil mixtures with the fungal isolate obtained from *A. sancta* roots, as well as with Tulasnella calospora (MK250656) from *S. vomeracea* and *Tulasnella helicospora* (MT612363) from *Anacamptis laxiflora*. Fifteen days after fungal inoculation, swelling occurred in both the control group seeds and the seeds inoculated with *T. calospora* and *T. helicospora*, with the seed coat rupturing; however, growth did not continue. Seeds inoculated with the Ceratobasidiaceae isolate obtained from *A. sancta* roots were incubated for 30 days. Of these seeds, 90.46% germinated and 16.98% developed into leafy and rooted seedlings. Twenty-one seedlings (stage 4-5) obtained with the *A. sancta* isolate (Ceratobasidiaceae) were transferred to the natural environment, and all developed their first true tuber.

In another study, conducted by Aytar & Kömpe (2024), *Serapias orientalis* seeds were successfully germinated using symbiotic methods and transferred to a natural area. Eighteen months later, the plants began to flower, marking the first successful reproduction of *S. orientalis* in the natural environment. Germination occurred in the packets 60 days after seed sowing, and the growth stages were determined. It was found that 62.58% of the seeds germinated, 16.93% formed protocorms, and 18.49% formed seedlings. No germination occurred in pots without fungi. In *S. vomeracea* seeds, a total germination rate of 82% was achieved. This rate is slightly lower than that of *A. sancta* but higher than that of *S. orientalis*. Both the differences in the fungi used and species differences can be cited as reasons for these variations.

Chlorophyll a and chlorophyll b values are compounds that are crucial for the plant's photosynthetic activity, and high values indicate that the plant can perform photosynthesis effectively. Furthermore, the high total chlorophyll content suggests the plant's effectiveness in growth and development processes. Beta-carotene and lycopene are essential carotenoids found in plants, possessing strong antioxidant properties. The presence of these values indicates that the plant has a high antioxidant capacity and can effectively combat free radicals (Melis & Harvey, 1981; Ebrahimi et al., 2023). In conclusion,

S. vomeracea seedling is an important plant in terms of antioxidant activity and contains various antioxidant components. These results can provide a foundation for the exploration and evaluation of the potential health benefits of the plant.

Various studies conducted on *S. vomeracea*, particularly the research by Acemi & Özen (2019), have demonstrated the successful initiation of germination under *in vitro* conditions. However, it has been observed in these investigations that the seedlings obtained did not reach full developmental maturity. The influential role of the nutrient mediums used, especially the control group, Kn (Knudson) 58.77 ± 5.51 environment, has been notably observed. In a separate study conducted by Acemi (2020), it was determined that the KN-11 medium exhibited the highest seed germination rate among indole acetic acid (IAA) applications, yet all tested indole-3-butyric acid (IBA) applications resulted in lower seed germination rates compared to the control group. Environments containing jasmonates (JAS), regardless of concentration, exhibited a reduction in germination rates when compared to the control group. The medium supplemented with chitosan oligomer mixture (CHI-OM) demonstrated a positive effect on seed germination, with the KN-21 medium recording the highest germination rate among these enriched environments. Environments supplemented with chitosan polymer (CHI-P) induced seed germination in all cases, and the highest germination rate was achieved in the KN-25 medium enriched with CHI-P (%74.97 ± 3.62). The protocol formation rate in the control group was documented as %7.45 ± 0.25. All treatments involving plant growth regulators (PGR) and chitosan significantly heightened the protocol formation rates. BAP (cytokinins [6-benzylaminopurine) and KIN (kinetin) applications resulted in comparable protocol formation rates, albeit with the highest rate observed in the KN-5 environment. Environments containing auxins exhibited statistically similar protocol formation rates, with the most favorable outcomes achieved in environments enriched with inductive auxins such as KN-11, KN-14, and KN-15. The addition of 1.0 mg L-1 JAS to the medium triggered the highest protocol formation rates. Among all tested environments, the KN-18 environment emerged as the most favorable with a protocol formation rate of %79.58 ± 2.92. The CHI-OM-enriched medium demonstrated a protocol formation pattern analogous to the medium enriched with JAS, with the KN-21 environment yielding the highest value (%79.02 ± 6.00), a proximity that is statistically significant. The CHI-P-containing medium resulted in lower protocol formation rates compared to other PGR and CHI-OM treatments. Notably, only the lowest CHI-P concentration yielded statistically similar results to the KN-2 medium (Acemi 2020). Our symbiotic *ex vitro* study on *S. vomeracea* indicates a notable increase in both total germination and seedling formation. In the investigation conducted by Kömpe et al. (2022), Tulasnella isolates were found to stimulate germination and growth at various rates. According to measurements taken over a three-month period, 98% of seeds in packages inoculated with the SVL 21 isolate germinated, and seeds inoculated with this isolate developed up to stage S3. Seeds in pots inoculated with Svl 4, Svl 14, and Svl 34 germinated at rates of 93.2%, 94%, and 90%, respectively, and these isolates supported seedling development up to stage S4 (13.67%, 10.8%, 9.8%, respectively). The percentage of seedlings reaching stage S4 (13.67%) inoculated with the Svl 4 isolate was statistically significantly higher than seedlings reaching the same stage inoculated with other fungi. In our study, we observed higher germination rates in various strains during the seedling development stage, with these results being determined as 52%.

The endangerment of numerous orchid species is, in part, a consequence of inherent rarity stemming from factors such as small population sizes, restricted distributions, and species-specific symbiotic relationships with pollen vectors and mycorrhizal fungi (Wraith et al., 2020) .This complexity gives rise to a nuanced ecological framework reliant on species-specific interactions and abiotic factors, susceptible to disruption by phenomena like climate change, habitat modification, and alterations in land use. Primary threats encompass invasive species impacts, changes in fire regimes, and illegal harvesting, collectively manifesting as threat syndromes and presenting the most prevalent challenges to orchids globally. Orchids, being charismatic entities with a historical allure for collectors, significantly contribute to population and species decline (Swarts & Dixon, 2009).

The effective conservation of orchids in their natural habitats is inherently challenging due to specific biotic factors and associated threat syndromes, necessitating interdisciplinary research efforts. Results from biochemical studies on *S. vomeracea*, as found in the literature, exacerbate the predicament by causing detrimental impacts on orchid populations protected by conservation measures, as individuals are frequently subjected to collection from natural habitats. Consequently, a strategic shift towards studies emphasizing sustainable agricultural practices, balancing both conservation and utilization objectives, becomes imperative (Aytar & Kömpe, 2023).

In the preceding study by D'Auria et al. (2021), biochemical investigations revealed the olfactory profile of *S. vomeracea*, with predominant constituents such as pentadecane (25.43%), 5-nonadecene (14.48%), and 1-nonadecene (5.16%). Additional aromatic components included 3-heptadecene (4.33%), benzyl benzoate (4.18%), 2-undecanone (3.31%), and octadecane (3.24%) (D'Auria et al., 2021a). In the study by Robusta De Culla et al. (2019), representative compounds in *S. vomeracea* encompassed pentacosane (17.59%), tricosane (14.21%), heneicosane (5.68%), heptacosane (4.99%), nonadecane (2.45%), tetracosane (1.90%), and tetradecane (1.68%). Monounsaturated linear hydrocarbons (18.63%) were identified, with aldehydes constituting a significant proportion (18.18%) of total volatiles, predominantly nonanal (7.87%), phenylacetaldehyde (3.91%), heptanal (1.57%), undecanal (1.35%), and octadecanal (0.93%).

Acidic compounds (1.92% of the total) were represented by palmitic acid (0.77%), nonanoic acid (0.71%), and heptanoic acid (0.37%). Terpenes (2.44%) accounted for trans-β-farnesene (0.84%), γ-muurolene (0.39%), and thymol (0.28%). The volatile fraction of *S. vomeracea* exhibited a singular alcohol, diacetone alcohol, comprising 1.03% of the total volatile oil.

Major compounds identified in the methanol extraction of *S. vomeracea* seedlings included tetracosamethyl-cyclododecasiloxane (28.83%), cyclononasiloxane, octadecamethyl- (10.51%), and hexanedioic acid, bis(2-ethylhexyl) ester (4.97%). The high content of DPPH and phenolic compounds is attributed to the influence of these major compounds. In the study conducted by Ertürk et al. (2023). ethanol extracts exhibited antimicrobial activity in both above-ground and tuber parts of *S. vomeracea*. In the same study, the phenolic content of above-ground parts was determined as 774.94 GAE/g extract, and the DPPH value was IC_{50}:13.33 (Ertürk et al. 2023). In our study, the results obtained indicated higher DPPH activity (IC_{50}:2.09) and lower total phenolic content (131.45 GAE/g extract).

This study aims to comprehensively investigate the *ex vitro* germination process of *S. vomeracea* seeds and the role of the SVL-30 in this process. The findings indicate that the SVL-30 significantly promotes seed germination and seedling development, marking a crucial step towards facilitating successful growth of *S. vomeracea* in its natural habitat. Furthermore, analyses conducted to determine the antioxidant properties of the plant reveal that *S. vomeracea* seeds are rich in phenolic compounds, exhibiting notable antioxidant activity. This underscores the potential health benefits and biological activity of the plant, emphasizing its significance for conservation efforts and ecological balance in natural habitats. Additionally, assessments of chlorophyll and carotenoid contents indicate that *S. vomeracea* maintains a healthy physiological status, highlighting its potential contribution to ecosystem stability and emphasizing the importance of its preservation in natural environments. Lastly, GC-MS analysis of methanol extracts identified various bioactive compounds, suggesting the potential contribution of *S. vomeracea* to biodiversity conservation and warranting further investigation in future research endeavours. This study lays a foundation for future studies focusing on the determination of plant biochemical profiles and bioactive compounds, potentially contributing positively to the preservation and sustainability of natural plant species.

Acknowledgements: This research was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) under the Project Grant No. 2209/A-1919B012204579. The authors gratefully acknowledge TÜBİTAK for their financial support.

Competing interests: The authors have declared that no competing interests exist.

Authors' contributions: Esin Çolak (Author 1): Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Erdi Can Aytar (Author 2): Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. Yasemin Özdener Kömpe (Author 3): Conceptualization, Supervisor, original draft, Writing – review & editing

Funding:

Funding agencies/institutions:	Project / Grant
Scientific and Technological Research Council of Türkiye	Scientific and Technological Research Council of Türkiye

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