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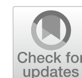

In vitro culture of the endangered plant *Eryngium viviparum* as dual strategy for its ex situ conservation and source of bioactive compounds

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Abstract

Different *Eryngium* species have been used with ornamental, agricultural and medicinal purposes, as a consequence of their chemical constituents. In the southwest Europe the endemic *Eryngium viviparum,* presents a high risk of extinction and ex situ strategies are high recommended for efficient conservation and re-introduction program. The objective of this study was to satisfy a dual objective: (i) to develop an ex situ conservation strategy through micropropagation and (ii) taking advantage of the extraordinary potential of plant tissue culture, produce a considerable amount of plant material to carry out a preliminary phytochemical study, based on the accumulation of phenolic compounds and their associated antioxidant activity. First a factorial design was conducted in order to study the effect of two cytokinins (6- benzylaminopurine, BAP, and kinetin, KIN), at three levels (0, 1 and 2 mg L^{-1}), on shoot multiplication. Later another factorial design was applied, by using three levels of MS medium salt strength (full, half and quarter- strength) and four sucrose levels (0, 1, 2, and 3%) for improving shoot elongation and rooting. In parallel, a preliminary quantification of total phenolic and flavonoid contents from *E. viviparum* aerial parts was determined. The simple micropropagation protocol designed allowed obtaining a high rates of shoot multiplication (5.1–5.8 new shoots), rooting (100%) with healthy long roots (3.1–3.5 cm) and plantlet acclimatization (96%). Moderate antioxidant activity was recorded in hydromethanolic extracts from *E. viviparum* aerial parts. High correlation between total phenolic content and BAP levels in the culture media was found. In conclusion, the micropropagation procedure described here for the endangered *E. viviparum* can be used as new and very efficient ex situ conservation strategy, and as potential source of antioxidants, conferring an added-value to this plant.

Key message

In this work, we addresses the development of an efficient in vitro culture procedure of *Eryngium viviparum* as ex situ conservation methodology, which leads to a plant reintroduction programs, and as new source for secondary metabolites (mainly phenolic compounds), without ecological impact in their limited populations (either using seeds or wild plants as source materials).

Keywords Apiaceae · Antioxidant activity · Endemic plant · Phenolic and flavonoids content · Phytochemistry · Threatened plant conservation

Introduction

Eryngium viviparum Gay, is a small, biennial and aquatic plant typical of Southwest Europe comprising France, Spain and Portugal. This species was classified as *vulnerable* by the International Union for Conservation of Nature (IUCN), in 1997 and included in the red list of threatened plants (Walter and Gillett 1998; Aguiar 2003; Romero et al. 2004). More recently, due to the reduction of their natural

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habitats and the consequent decrease in the number of plants (Bañares et al. 2004), its classification has been moved to *endangered* (Lansdown 2011). In fact, this reduction is due to much of anthropogenic habitat destruction that cause species extinction, loss of genetic diversity and destruction of biological communities, which are vital to ecosystem functioning and human welfare (Silveira et al. 2016).

Management of wild populations and protection of natural habitats are usual by in situ conservation strategies used for the protection of threatened plants. However, in critical situations, ex situ strategies such as in vitro techniques, cryopreservation or storage of germplasm are necessary (Sarasan et al. 2006). Plant in vitro culture is a biotechnological tool, which offers a plethora of applications for plant conservation. This technique has several advantages such as high rates of propagation, production of disease-free plants or germplasm storage. Furthermore, the high amount of plants produced by this technique allows the establishment of plant reintroduction programs or the use of in vitro-cultured plants with research purposes, without ecological impact on their limited natural populations (González-Benito and Martín 2011). Micropropagation has been successfully applied as an ex situ conservation strategy for many threatened plants (Fay 1992; Engelmann 2011). In fact, it was recommended for *E. viviparum* in the Atlas and Red Book of Spanish Threatened Vascular Flora (Bañares et al. 2004) although it has not been implemented to date.

Furthermore, plant in vitro culture constitutes a great biotechnological tool for the study of plant secondary metabolism due to the improvement on the disadvantages attributed to conventional plant breeding, such as low growing rate and low production yields of secondary metabolites, emerging as an efficient system for bioactive compound production (Karuppusamy 2009; Dias et al. 2016; Tusevski et al. 2017; Isah et al. 2018; Hu et al. 2019).

The *Eryngium* genus, like many other members of Apiaceae family, has been used with ornamental, agricultural and medicinal purposes, as a consequence of their chemical constituents, which have been studied in terms of their phytochemical and pharmacological activities (Wang et al. 2012; Erdem et al. 2015). Hence, phenolic compounds have been highlighted as one of major compounds of *Eryngium* genus (Küpeli et al. 2006). These compounds found in plant extracts exert potent antioxidant and cytotoxic activities against different cancer cell lines, thus revealing beneficial properties for cancer therapy and prevention (Belkaid et al. 2006; Yip et al. 2006). Moreover, phenolics have gained much attention due to their additional bioactivities, as astringent, antiviral, antibacterial and anti-inflammatory agents (Petersen and Simmonds 2003; Gugliucci and Bastos 2009). Previous studies have confirmed the presence of these antioxidant compounds in *Eryngium* genus in both field-grown (Le Claire et al. 2005; Cádiz-Gurrea et al. 2013) and in vitro- cultured plants (Kikowska et al. 2012; Thiem et al. 2013). However, the information about the phytochemical compounds and bioactivities in *E. viviparum* is unknown.

In this research paper, we have developed the first micropropagation protocol for *E. viviparum*, as the starting point for the establishment of efficient ex situ conservation and reintroduction strategy. Moreover, we carried out a preliminary study using in vitro-cultured plants and the quantification of total phenolic and flavonoid contents from *E. viviparum* aerial parts was determined. Such compounds were correlated to their antioxidant activity, as they act as free radical scavenging agents. Altogether, our results may be highly useful for the establishment of further strategies for the study of the phytochemical potential of *E. viviparum.*

Materials and methods

Plant material

Mature brown fruits (schizocarps) of *Eryngium viviparum Gay* were collected on the margin of the Cospeito Lake, Lugo, Spain (43°14′30.16″N, 7°32′55.539″W). These fruits were kept in dry paper bags under room laboratory conditions until use. Individual mericarps (seeds) were obtained by mechanical friction and stored in Petri dishes at 4 °C until use. Seeds were disinfected and germinated in vitro, as described previously (Ayuso et al. 2017). Briefly, seeds were first soaked in 2% sodium hypochlorite for 5 min and washed with sterile distilled water (three times). After, seed were stirred in 50% sulphuric acid for 40 min, removed carefully and washed, in sterile distilled water, during 5 min (three times) and soaked overnight.

Micropropagation

Culture media and conditions

Culture media consisted in MS medium (Murashige and Skoog 1962) solidified with 1% agar (w/v) and supplemented with 3% sucrose (w/v) at pH 5.8. All media were autoclaved at 121 °C for 20 min at 105 kPa and plant cultures were incubated at 24 ± 1 °C under a photoperiod of 16 h light and 8 h dark in a growth chamber (flux density: 40 µmol m^{-2} s^{-1}).

Establishment and shoot multiplication stages

Seedlings from in vitro germination (Ayuso et al. 2017) were established on MS medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) and 0.1 mg L^{-1} indole-3-butyric acid (IBA). Plantlets were maintained for 2 subcultures (30 days each one), under the conditions described above,

until obtaining an adequate number of plantlets for the next step. Percentage of survived plants at the end of the establishment stage were recorded.

Established plantlets were placed for multiplication in vessels containing 25 mL of MS medium supplemented with 0.1 mg L^{-1} IBA combined with two cytokinins (BAP and kinetin; KIN). A factorial design was applied to study the effect of both cytokinins (BAP and KIN) at three levels (0, 1 and 2 mg L^{-1}), then a total of 9 treatments (3 BAP levels × 3 KIN levels; named T1–T9) were tested. Shoots clusters formed during this stage were divided into single shoots and subcultured every 5 weeks during eight subcultures. New shoot number and shoot length (SN and SL, respectively) were recorded in the last four subcultures (5th to 8th) for each explant. Each treatment consisted of six culture vessels sealed with plastic caps, containing three plantlets each one. The experiments were carried out in triplicate.

Shoot rooting and acclimatization stages

Single shoots from the fifth multiplication subculture were transferred to three different MS-based media supplemented with four different sucrose concentrations and 0.1 mg L^{-1} IBA. A factorial design was applied, including three levels for MS salt strength (1, 0.5 and 0.25) and four levels for sucrose concentration (0, 1, 2, and 3%). Then, 12 treatments (3 MS strengths × 4 sucrose concentrations; named R1–R12) were tested. The initial and final shoot length (after 30 days) were recorded in order to calculate the increase of shoot length (ISL). In addition, root length (RL) and root dry weight (RDW) were determined after 30 days using five plants per treatment and repeated thrice. RDW was achieved after drying fresh roots at 60 °C until continuous weight.

Healthy elongated plantlets (100) with well-developed roots were placed in pots with a mixture of peat and perlite (1:1 v/v) for acclimatization. They were covered with plastic boxes and placed in a growth chamber with humidity control for 20 days. Relative humidity was ranged from 100 to 70%, subtracting 10% every 5 days. Survival frequency was recorded at the end of the acclimatization stage.

Healthy rooted plantlets were transferred to greenhouse first for hardening and later to their natural habitat at the Cospeito Lake, Lugo (NW Spain; Ayuso et al. 2017).

Evaluation of antioxidant activity and phenolic compounds

Extraction

Aerial parts from the 5th to 8th subcultures (multiplication stage) were excised and stored at – 20 °C. They were frozen-dried and powdered to get a homogeneous material. The extraction procedure was based on the work developed by Ali et al. (2013). All extractions were performed three times.

Briefly, 100 mg of frozen-dried plant material was subjected to phenolic extraction, using 10 mL of methanol:water (80:20), incubated at 60 °C in a water bath for 10 min and later sonicated for 30 min in the dark. The hydromethanolic extracts were filtered using glass microfiber filters (1.2 µm pore size) and stored at – 20 °C. Milli-Q grade water was used in all biochemical determinations and all reagents were analytical grade. Hydromethanolic extracts were used for the subsequent determinations.

Total phenolic content determination

Total phenolic content (TPC) was determined through Folin Ciocalteu´s method applied to plant tissues as described by Ainsworth and Gillespie (Ainsworth and Gillespie 2007). Briefly, 100 µL of hydromethanolic extracts were mixed with 200 µL of 10% Folin Ciocalteu´s reagent and 800 µL of 0.7 M sodium carbonate. The samples were vortexed and incubated for 2 h in the dark at room temperature. The absorbance was measured at 765 nm using UV–visible spectrophotometer against a blank containing a solvent, instead of a sample. A calibration curve with gallic acid (0–1000 mM) as standard was performed. Results were expressed as gallic acid equivalents in mg per gram of dry weight (mg GAE g^{-1} DW). All measurements were carried out in triplicate.

Flavonoid content determination

Flavonoid content from hydromethanolic extracts was studied by the method developed by Pekal and Pyrzynska (2014). Briefly, 1 mL of extracts were mixed with 0.5 mL of 2% aluminum chloride and 0.5 mL of milli-Q water. The samples were vortexed and incubated for 10 min at room temperature in the dark. Absorbance was measured at 425 nm against a blank containing solvent. Quercetin was used as the standard for the calibration curve (0–150 µM) and the results were expressed as quercetin equivalents in mg per gram of dry weight (mg QE g^{-1} DW). All measurements were carried out in triplicate.

Evaluation of antioxidant activity

Antioxidant activity of plant extracts was analyzed through DPPH method (1,1-diphenyl-2-picrylhydrazyl) described by Brand-Williams et al. (1995) and modified by Thaipong et al. (2006). A 0.6 mM DPPH stock solution was prepared in methanol and stored at – 20 °C. Next, a 0.1 mM of DPPH working solution (WS) was prepared daily from a stock solution in the same solvent. Briefly, 150 µL from plant extracts were mixed with 2850 µL of DPPH WS and incubated for

30 min in the dark at room temperature. Absorbance was measured at 517 nm using UV–Vis spectrophotometer against a blank containing the solvent, instead of a sample. The results were expressed as inhibitory concentration 50 (IC50), given by the inhibition percentage of DPPH in the presence of plant extracts, which represents the extract concentration (mg DW mL^{-1}) needed to reduce by 50% the free-radical activity caused by DPPH. All measurements were carried out in triplicate.

Statistical analysis

The collected continuous data were subjected to one-way ANOVA analyses, followed by Tukey HSD post hoc test. Count data as SN should be analyzed through Poisson regression (Agresti 1996) although, if there are more than 10 data, ANOVA and Poisson regression had the same inference (Mize et al. 1999). Thus, count data were also analyzed using one-way ANOVA and Tukey HSD post hoc test. Correlation between cytokinins concentrations, TPC, FC and IC50 were analized by Pearson´s correlation. All analyses were conducted using STATISTICA v. 12 software (StatSoft, Inc. 2014).

Results and discussion

In vitro culture establishment and shoot multiplication

Establishment of plant material is the first stage in micropropagation procedures. This stage is successfully accomplished when the contaminant-free explant development in the culture medium is achieved (George et al. 2008). Our results show that all seedlings (100%) were established in MS medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IBA. In addition, microbial contaminants were not observed since all seedlings established proceeded from in vitro germination. Although the establishment stage is typically very short (10–14 days) here, due to very slow growth of eryngium rosette it was extended for two subcultures of 30 days each.

In vitro shoot multiplication is the second stage of micropropagation with the aim of obtaining new propagules or shoots which may lead to new fully developed plants. The presence of one or more cytokinins within the culture medium is necessary for a successful multiplication stage. Hence, these phytohormones cause a reduction in apical dominance, thus enabling the emergence of new shoots (George et al. 2008). BAP and KIN are the main cytokinins used as plant growth regulators (PGR) on in vitro cultures (Gaspar et al. 1996).

In order to study the effect of these cytokinins on shoot multiplication, a factorial design with three cytokinins concentration levels (0, 1 and 2 mg L^{-1}) was followed. New shoot number (SN) and shoot length (SL) were measured for each treatment (Table 1).

The lowest SN values were recorded in the cytokinin-free medium and in media supplemented only with KIN (T1–T3; Table 1). On the contrary, exogenous addition of BAP alone in the MS medium, T4 and T7, showed a significant increase on SN values: 1.6 and 4.6 respectively. These values are significantly higher than T1–T3 SN values. The effective concentration of a particular cytokinin is specific for each species, variety and tissue or organ culture (Vieitez and Vieitez 1980; George et al. 2008; Máximo et al. 2018). In previous studies (Thiem et al. 2013; Kikowska et al. 2016), 1 mg L^{-1} BAP was enough to induce the highest SN (17, 13 and 4.4) in *E. planum, E. campestre* and *E. maritimum* respectively. Futhermore, Chandrika et al. (2011) described that higher BAP concentrations are needed (2–3 mg L^{-1}) to achieve the highest SN in *E. foetidum* (3.1–3.7). These large differences between SN are due to the specific shoot development in vitro of each species. *E. viviparum* grows exclusively as a rosette under in vitro conditions (Fig. 1a), as it is the case of *E. maritimum* and *E. foetidum*, which tend to form less shoots in the same conditions (Chandrika et al. 2011; Kikowska et al. 2014). Therefore, *E. viviparum* showed similar SN values, ranging from 1.6 to 4.6 (Table 1) than those species with similar in vitro development (3.1–4.4).

SN production was improved in media supplemented with BAP and KIN in combination. The highest concentration of BAP alone (2 mg L^{-1}) produced an average of 4.6 new shoots, higher than any other treatment with individual cytokinin content (Table 1). However, this value was improved significantly to 5.8 and 5.1 when any KIN concentration

Table 1 Effect of BAP and KIN (mg L^{-1}) on new SN and SL (cm) during in vitro *E. viviparum* multiplication stage

Treatment	BAP (mg L^{-1})	KIN (mg L^{-1})	SN	SL (cm)
T1	0	0	0.6 ± 0.1^{f}	2.2 ± 0.1^{a}
T2	0	1	0.1 ± 0.1^{f}	2.0 ± 0.1^{a}
T3	0	2	0.1 ± 0.1^{f}	1.6 ± 0.2^{a}
T4	1	0	1.6 ± 0.3^{e}	2.6 ± 0.1^{a}
T5	1	1	3.8 ± 0.3^{cd}	2.3 ± 0.1^{a}
T6	1	2	3.1 ± 0.3^{d}	2.5 ± 0.1^{a}
T7	2	0	4.6 ± 0.3^{bc}	2.4 ± 0.3^{a}
T8	2	1	5.8 ± 0.3^{a}	1.9 ± 0.2^{a}
T9	2	2	5.1 ± 0.3^{ab}	2.0 ± 0.1^{a}

Average of four subcultures (5th to 8th) data is shown

Mean values (± standard error) within the same column with different letters are significantly different at $p < 0.05$ using Tukey's post hoc multiple comparison test

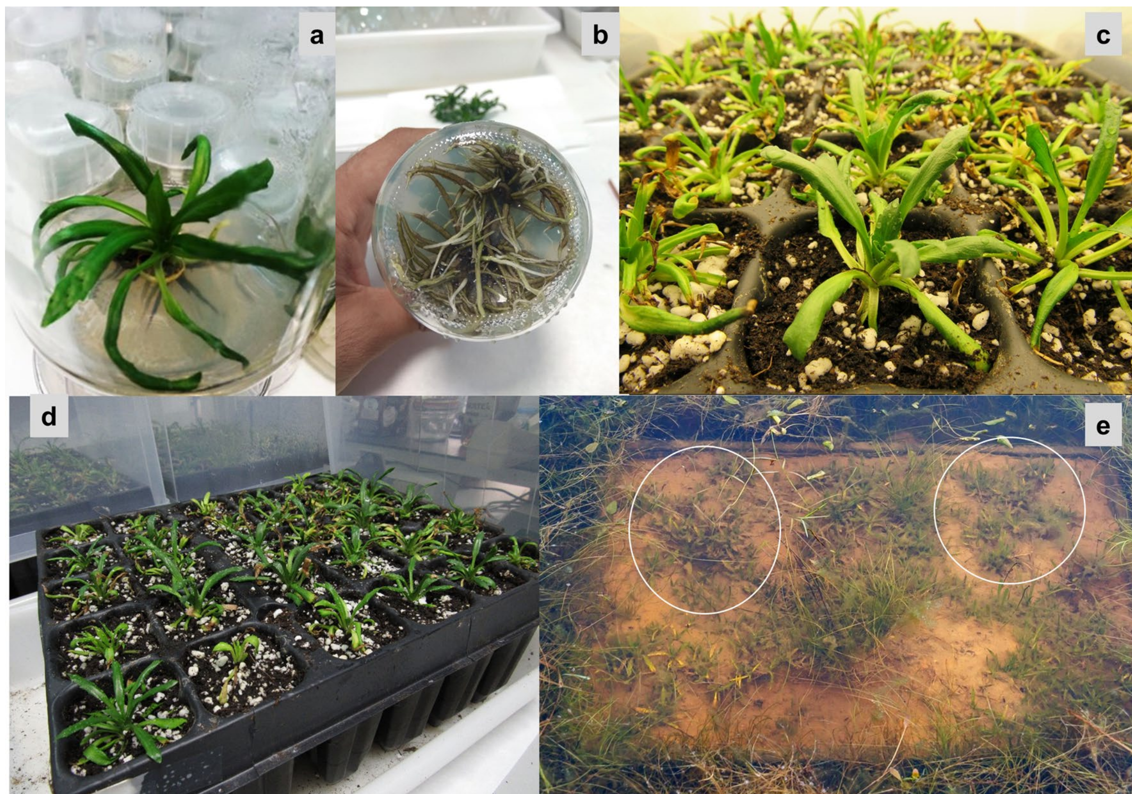

Fig. 1 In vitro-cultured *E. viviparum* showing the typical rosette development on the multiplication stage (**a**). New roots formed after 30 days on rooting stage (**b**). *E. viviparum* plantlets placed in pots with peat and perlite (1:1 v/v) substrate and covered with plastic boxes during acclimatization stage (**c**). *E. viviparum* plantlets successfully transferred to *ex vitro* conditions and ready for reintroducing in its natural habitat (**d**). *E. viviparum* successfully reintroduced at the Laguna de Cospeito, Lugo (NW Spain) (**e**)

was combined with 2 mg L^{-1} BAP, T8 and T9, respectively (Table 1). Thus, KIN seems to have a positive effect in combination with BAP on SN formation in *E. viviparum* micropropagation as it was reported for *E. foetidum* (Gayatri et al. 2006; Chandrika et al. 2011).

Additionally, high concentrations of cytokinins and long-term cultures may lead to the presence of very small and/or hyperhydric shoots, which is not desirable for plant in vitro culture (Debergh et al. 1992; George et al. 2008). However, in our case, no hyperhydric shoots were found in long-term cultures (at least until the eighth subculture). Nevertheless, no significant effect of cytokinins on SL was detected as compared to free-cytokinin medium (Table 1).

In vitro rooting and acclimatization stages

Rooting constitutes the third stage on micropropagation protocols and it is essential for the correct development of newly formed plantlets during the multiplication phase, as this lack an effective root system. For such purpose, auxins, and more specifically IBA, are commonly used as inductors of root formation (George et al. 2008). In this sense, many species belonging to Apiaceae family have been successfully rooted under the administration of IBA such as *Anethum graveolens* (Sharma et al. 2004), *Thapsia garganica* (Makunga et al. 2003) and *Vanasushava pedata* (Karuppusamy 2009). Nevertheless, the presence of IBA is not essential for root formation, since sucrose and medium salt concentrations usually show a strong effect on the induction of this process, as reported for other *Eryngium* species (Thiem et al. 2013; Kikowska et al. 2014, 2016). However, the effect of salt and sucrose concentration on rooting has never been studied in *E. viviparum*.

In our case, new-formed shoots from the fifth subculture of multiplication stage were subjected to rooting, using three different MS salt concentrations in combination with four different sucrose concentrations, all of them supplemented with 0.1 mg L^{-1} IBA.

Our results showed that new roots were formed in all shoots cultured in every rooting media after 30 days (Fig. 1b). The absence of sucrose inhibits root growth since the lowest RL values were recorded in sucrose-free media (R1, R5 and R9; Table 2). Conversely, higher RL values were reported with increased sucrose concentrations (Table 2) and consequently, the combination of 0.1 mg L^{-1} IBA, the presence of sucrose and higher salt concentrations

Table 2 Effects of MS salt strength (full = 1; half = 0.5; quarter = 0.25) and sucrose concentration (% w/v) on increase of shoot length (ISL), root length (RL) and root dry weight (RDW) during in vitro *E. viviparum* rooting stage

Treatment	Salt (strength)	Sucrose (% w/v)	ISL (cm)	RL (cm)	RDW (g)
R1	1	0	0.4 ± 0.1^{e}	1.3 ± 0.1^{e}	0.01 ± 0.01^{e}
R2	1	1	2.1 ± 0.2^{ab}	2.8 ± 0.1^{cd}	0.07 ± 0.01^{d}
R3	1	2	2.7 ± 0.2^{a}	3.5 ± 0.2^{ab}	0.11 ± 0.03^{c}
R4	1	3	2.2 ± 0.3^{ab}	3.7 ± 0.1^{a}	0.13 ± 0.02^{c}
R5	0.5	0	0.3 ± 0.1^{e}	1.4 ± 0.1^{e}	0.01 ± 0.01^{e}
R6	0.5	1	1.5 ± 0.2^{bc}	3.2 ± 0.1^{abc}	0.13 ± 0.01^{c}
R7	0.5	2	2.1 ± 0.2^{ab}	3.1 ± 0.1^{abc}	0.24 ± 0.01^{a}
R8	0.5	3	1.1 ± 0.1^{cd}	3.3 ± 0.1^{abc}	0.18 ± 0.01^{b}
R9	0.25	0	0.2 ± 0.1^{e}	1.6 ± 0.1^{e}	0.02 ± 0.01^{e}
R10	0.25	1	0.4 ± 0.1^{e}	2.9 ± 0.1^{cd}	0.06 ± 0.01^{d}
R11	0.25	2	0.3 ± 0.1^{e}	2.5 ± 0.1^{d}	0.07 ± 0.01^{d}
R12	0.25	3	0.6 ± 0.1^{de}	3.0 ± 0.1^{bcd}	0.06 ± 0.02^{d}

Mean values (± standard error) within the same column with different letters are significantly different at $p < 0.05$ using Tukey's post hoc multiple comparison test

(full and half strength) was successful for root growth (R3, R4, R6, R7, R8; Table 2). However, only T7 promoted a significantly larger root system because of its RDW value.

In *E. campestre* the best rooting (longest roots and higher root dry biomass) was produced using full-strength MS medium (1) with 5% sucrose (Kikowska et al. 2016) but for *E. maritimum* the best rooting was achieved in half-strength MS medium (0.5) supplemented with 1.5% of sucrose (Kikowska et al. 2014). Our results suggest that the best rooting medium was obtained in R7 combining half-strength MS with 2% sucrose (Table 2). Therefore, the influence of salt and sugar concentrations used for in vitro rooting media seems to be specific for each species.

In parallel, during this stage, shoot growth was influenced by salt and sucrose concentrations. Full-strength media in the presence of sucrose or half-strength medium with 2% sucrose showed the highest ISL values (R2–R4 or R7, respectively; Table 2). Therefore, our results suggest that root and shoot development are influenced by salts and sucrose concentration. These findings must be used as the starting point for future studies on *E. viviparum* in vitro propagation such as culture media optimization.

Acclimatization of in vitro-grown plants to *ex vitro* conditions is the last stage in micropropagation procedure. This acclimatization process is a crucial stage because if not carried out carefully, a high amount of propagated plants could be lost (George et al. 2008). In vitro plantlets facing acclimatization need suitable substrate (such as peat and perlite) to get an efficient root development in the new conditions and the residence under certain physical conditions including humidity, which subsequently will be reduced for intervals, and temperature (Sutter and Langhans 1982; Marín and Gella 1987; George et al. 2008). *E. viviparum* plantlets from in vitro culture were successfully acclimatized to *ex vitro* conditions with 96% of survived plants (Fig. 1c, d), in agreement with previous results obtained for *E. maritimum* 90% and *E. planum* 89% (Thiem et al. 2013; Kikowska et al. 2014). Acclimatized plants were successfully transfer to their natural environment (Fig. 1e).

Phenolic compound determination and evaluation of RSA

Phenolic compounds constitute the largest family within plant secondary metabolites, including more than 8000 different compounds. Amongst the different bioactivities associated with this compound family, phenolics have been reported as major antioxidant agents owing to their structural characteristics and chemical behavior. Thus, due to their hydrogen-donating ability, phenolic compounds may act as free-radical scavengers and, consequently, exert a protective effect against these highly reactive, oxidizing species (Rice-Evans et al. 1995, 1996; Nicole Cotelle 2001; Dai and Mumper 2010).

We conducted a preliminary study concerning the determination of total phenolic content (TPC), flavonoid content (FC) and free-radical scavenging, using hydromethanolic extracts from *E. viviparum* in vitro-derived aerial parts. Furthermore, the effect of cytokinins on TPC and FC as well as their effect on free radical scavenging activity were determined.

The basal levels of TPC and FC in aerial parts from in vitro-cultured *E. viviparum* were recorded in the cytokinin-free medium, T1: 18.1 mg GAE g^{-1} DW and 4.66 mg QE g^{-1} DW, respectively (Table 3).

All media supplemented with cytokinins caused an increase in TPC, in comparison to the cytokinin-free medium (Table 3). In fact, the highest TPC value was recorded in the media supplemented with the highest concentrations of both cytokinins (T9, Table 3). This value (34.8 mg GAE g^{-1} DW) was similar to those found in other medicinal plants, e.g. Miliauskas et al. (2004) determined the TPC in methanolic

Table 3 Effects of KIN and BAP (mg L^{-1}) on total phenolic content (TPC), flavonoid content (FC) and inhibitory concentration of 50% DPPH (IC50) on in vitro *E. viviparum* aerial parts from the last four subcultures

Treatment	KIN (mg L^{-1})	BAP (mg L^{-1})	TPC GAE (mg g^{-1} DW)	FC QE (mg g^{-1} DW)	DPPH IC50 (mg mL^{-1})
T1	0	0	18.1 ± 0.5^{e}	4.66 ± 0.09^{de}	4.76 ± 0.20^{d}
T2	0	1	31.4 ± 0.2^{b}	5.04 ± 0.05^{cd}	2.84 ± 0.04^{ab}
T3	0	2	29.6 ± 0.1^{bc}	4.94 ± 0.08^{cde}	2.63 ± 0.08^{a}
T4	1	0	24.8 ± 0.7^{d}	4.56 ± 0.05^{e}	3.42 ± 0.08^{c}
T5	1	1	29.3 ± 0.6^{bc}	5.32 ± 0.17^{c}	3.20 ± 0.04^{bc}
T6	1	2	27.5 ± 0.8^{c}	6.76 ± 0.06^{b}	2.98 ± 0.04^{ab}
T7	2	0	24.6 ± 0.9^{d}	3.72 ± 0.04^{f}	3.04 ± 0.02^{bc}
T8	2	1	29.5 ± 0.5^{bc}	6.63 ± 0.08^{b}	2.68 ± 0.01^{a}
T9	2	2	34.8 ± 0.2^{a}	7.68 ± 0.10^{a}	2.64 ± 0.03^{a}

Mean values (± standard error) within the same column with different letters are significantly different at $p < 0.05$ using Tukey's post hoc multiple comparison test

extracts from 12 medicinal plants and this content ranged between 4.1 and 37.9 mg GAE g^{-1} DW.

In the same way, concerning FC, the highest FC concentration, 7.68 mg QE g^{-1} DW, was recorded in the 2 mg L^{-1} combination of both cytokinins (T9; Table 3). However, BAP and KIN alone did not improve the basal value of the cytokinin-free medium. This value was considerably lower than the values found in methanolic extracts from other medicinal plants, obtaining FC values between 3.67 and 648.67 mg QE g^{-1} DW (Agbo et al. 2015).

Then, BAP and KIN may have an elicitor effect on the production and accumulation of phenolic compounds in the aerial parts from in vitro-cultured *E. viviparum*. Cytokinins can act as elicitors on the biosynthesis of cinnamic acid, which is the common precursor of most polyphenols (Treutter 2010; Dias et al. 2016).

Additionally, the antioxidant activity of *E. viviparum* hydromethanolic extracts was evaluated through their radical scavenging activity, RSA, using the stable free-radical DPPH. When dissolved in methanol, DPPH presents a characteristic violet color, which is inhibited by the addition of free-radical scavenging agents in the reaction mixture. (Villaño et al. 2007). These extracts were recorded by the inhibitory concentration 50 (IC50), which constitutes the extract concentration needed to inhibit, by 50%, the absorbance due to DPPH. It is important to note that lower IC50 values imply a higher antioxidant activity since lower extract concentrations are needed to achieve IC50. The extract concentration required for IC50 was significantly lower in BAP and KIN treatments, compared to cytokinin-free medium, T1 (Table 3). Once again, T9 (together with other cytokinin containing treatments) promoted the lowest IC50 values and therefore, the highest antioxidant activity (Table 3). These values supposed to exert a moderate RSA compared to other medicinal plants, e.g. Mongkolsilp et al. (2004) recorded the IC50 values of methanol extracts from six medicinal plants and IC50 values ranged from 0.006 to 23.1 mg mL^{-1}.

Pearson´s correlation showed a strong effect between TPC and IC50 values ($p < 0.001$; Table 4). This correlation was negative since a higher concentration of TPC correlates to lower IC50 concentration (Table 4). In addition, this correlation showed a strong positive effect between BAP and TPC and negative in BAP and IC50. KIN and FC did not reveal a significant correlation on IC50 concentration (Table 4). Thus, media supplemented with BAP increased TPC in extracts from aerial parts of *E. viviparum* and consequently, improved their RSA, by decreasing the IC50 values.

The most powerful antioxidant phenolic compounds are flavonoids (especially the flavanols) and phenolic acids (Matkowski 2008). In *Eryngium* species were identified several phenolic acids with powerful antioxidant properties, such as chlorogenic, caffeic and rosmarinic acids (Le Claire et al. 2005; Wang et al. 2012). The latter was found in high concentration on in vitro cultures of *E. maritumum* and *E. planum* (Thiem et al. 2013; Kikowska et al. 2014). Consequently, the negative correlation between TPC and IC50 and the non-significant correlation with FC, could be due to the presence of other non-flavonoids compounds, in the hydromethanolic extracts of *E. viviparum*, with antioxidant properties (Table 4).

Table 4 Pearson´s correlation coefficients for cytokinins (BAP and KIN), total phenolic content (TPC), flavonoid content (FC) and inhibitory concentration for 50% of DPPH (IC50) on in vitro *E. viviparum* aerial parts from the last four subcultures

	KIN	BAP	TPC	FC
TPC	0.30^{NS}	0.74**		
FC	0.38^{NS}	0.73**	0.62*	
IC50 (DPPH)	-0.4^{NS}	− 0.64*	− 0.87***	-0.42^{NS}

[NS]No significant differences at $p \geq 0.05$; *Significant differences at $p < 0.05$; **Significant differences at $p < 0.01$; ***Significant differences at $p < 0.001$

Conclusion

In conclusion, we described here the first *E. viviparum* micropropagation protocol. This protocol allowed repopulating its damaged habitats with a large number of plants, by constituting an efficient ex situ conservation strategy. Additionally, this preliminary study provides insight about the phenolic content of in vitro-cultured *E. viviparum* and its antioxidant activity added-value to this *endangered* plant. BAP appears to play an important role in the production and accumulation of these phenolic compounds on the in vitro aerial parts of *E. viviparum*.

Finally, future studies based on the phytochemical analysis of *E. viviparum* should be focused on the development of phenolic profiling, including the identification of the main compounds responsible for its antioxidant activity. This characterization would lead to a wider analysis, which could be applied to different organs, such as roots, thus enabling a deep knowledge about the pharmacognostical properties of this species, unraveling its potential for industrial applications.

Author contributions MA: Performed micropropagation experiments; MA and PGP: Performed phenolic compound determination and evaluation of RSA; PR-R: Contributed with plant seeds, reagents and materials; MA, PPG and MB: Conceived and designed the experiments. All authors contributed to the writing of the manuscript.

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Compliance with ethical standards

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

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