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In vitro culture of Eryngium viviparum: An endangered plant with therapeutic phytochemical potential

THESIS DISSERTATION

Manuel Ayuso Vilaboa

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DOCTORAL DISSERTATION

In vitro culture of *Eryngium viviparum:* An endangered plant with therapeutic phytochemical potential

Supervised by:

Dr. Mª. Esther Barreal and Prof. Dr. Pedro Pablo Gallego

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DECLARE that the present work, entitled "***In vitro* culture of *Eryngium viviparum*: An endangered plant with therapeutic phytochemical potential"**, submitted by Mr. Manuel Ayuso Vilaboa (BsC, MsC) to obtain the Doctoral degree with International Mention and by Compendium of Publications, was carried out under their supervision in the PhD Program "Advanced Biotechnology".

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The supervisors

Dr. Mª Esther Barreal **Prof. Dr. Pedro Pablo Gallego**

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Publications

Journal articles

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3. **Ayuso, M.**, Pinela, J., Dias, M.I., Barros, L., Ivanov, M., Calhelha, R.C., Sokovi , M., Ramil-Rego, P., Barreal, M.E., Gallego, P.P., Ferreira, I.C.F.R., 2020. Phenolic composition and biological activities of the *in vitro* cultured endangered *Eryngium viviparum* J. Gay. Ind. Crops Prod. 148, 112325. https://doi.org/10.1016/J.INDCROP.2020.112325

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1. **Ayuso, M.**, De Souza, M.A., Domínguez, S., Gomez, L., Rodríguez, R., Covelo, E., Pedrol, N. 2013. Ecophysiological interactions of water and cd stresses in *Brassica juncea.* XIII Congresso Luso-Espanhol de fisiologia vegetal. Poster. Lisbon, Portugal.

2. **Ayuso, M.**, Gómez, B., Ramil, P., Gallego, P.P., Barreal, M.E. 2016. Micropropagation of *Eryngium viviparum* J. Gay: a New Way to Recover this Endangered Plant with Potential in Pharmacology. First European Conference of Post Graduate Horticulture Scientists. Poster. Palermo, Italy.
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4. Verde, A., **Ayuso, M.**, González, B., Míguez, J.M., Gallardo, M. 2017. Role of melatonin in L-tryptophan-induced benefits on water stress during early-growth stages of Cicer arietinum L. Green for Good IV: Biotechnology of plant products. Poster Olomouc, Czech Republic.
5. **Ayuso, M.**, Pinela, J., Dias, I.D., Gallego, P.P., Barreal, M.E., Barros, L., Ferreira, I. 2018. Phenolic composition and cell-based antioxidant activity of roots and aerial parts of *Eryngium viviparum* produced *in vitro*. XXIV Encontro Luso-Galego de Química. Oral. Porto, Portugal.
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7. **Ayuso, M.**, Dias, I.D., Pinela, J., Gallego, P.P., Barreal, M.E., Barros, L., Ferreira, I. 2019. Valorization of a threatened plant species through *in vitro* culture elicitation of phenolic compounds. Congreso Nacional de Biotecnología (BIOTEC). PosterVigo, Spain.

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"Sometimes you climb out of bed in the morning and you think, I'm not going to make it, but you laugh inside — remembering all the times you've felt that way"

Charles Bukowski

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Resumen

Resumen

Las plantas son esenciales para muchos procesos vitales en la tierra; sin embargo, en los últimos años, su tasa de extinción ha alcanzado niveles nunca antes observados por la comunidad científica. Además de los muchos procesos clave para la vida en los que están involucradas, las plantas son fuente de múltiples metabolitos secundarios que pueden tener gran interés para el ser humano. Estos, son compuestos de bajo peso molecular que se sintetizan en bajas concentraciones a través de diferentes vías metabólicas y están involucrados en la defensa de la planta, resistencia al estrés y en su interacción con el entorno circundante. Además, estos metabolitos se han asociado con múltiples actividades biológicas importantes para la salud humana y su bienestar. Así, las plantas ricas en compuestos bioactivos se han utilizado durante décadas en la medicina tradicional y, más recientemente, se han aplicado en diferentes sectores, como la industria alimentaria, farmacéutica y cosmética. Por tanto, las acciones coordinadas para la conservación de especies vegetales son una estrategia fundamental, tanto para preservar procesos vitales en los que están involucradas y que benefician directamente a la vida en la tierra, como para poder ser explotadas como fuentes alternativas y valiosas de compuestos bioactivos.

Las medidas de conservación más utilizadas y eficaces son las que aseguran la protección de las especies vegetales dentro de su hábitat original. Estas medidas se denominan estrategias *in-situ* y permiten la preservación, a través de la protección de diversas regiones de particular interés, preservando las interacciones de las especies amenazadas con el resto del ecosistema. Debido a que estas estrategias pueden no ser efectivas, existen enfoques complementarios que ayudan a conservar la diversidad genética de la especie a medio y largo plazo en poblaciones con muy pocos individuos, que se encuentran en alto riesgo. Estas estrategias se denominan *ex-situ* y permiten preservar el material genético heterogéneo de poblaciones en peligro fuera de su hábitat natural. Generalmente, los jardines botánicos son los principales encargados de las estrategias *ex-situ* mediante la creación de bancos de semillas y colecciones vivas de estas especies. Adicionalmente, la aplicación de

técnicas de cultivo *in vitro* es necesaria en ciertos casos amenazados para asegurar la conservación de una cantidad adecuada de germoplasma.

Eryngium viviparum es la especie de estudio en la presente tesis. Esta especie es una planta amenazada perteneciente a la familia Apiaceae distribuida por humedales del noroeste de la Península Ibérica y Francia. Durante el invierno, esta especie vive sumergida dentro de estanques o lagos, y en verano cuando el nivel del agua disminuye, debido a las altas temperaturas, queda expuesta. Durante ese período, puede reproducirse sexualmente a través de estolones con formación de flores, que luego darán lugar a semillas y nuevas plántulas. Esta especie está considerada como "endangered" por la Unión Internacional para la Conservación de la Naturaleza (UICN) y está incluida en su lista roja de especies amenazadas. Además, el Libro Rojo de la Flora Vascular Amenazada en España también incluye este endemismo. Ambas recopilaciones sobre el estado de amenaza de la especie coinciden en la necesidad de implementar medidas de conservación *ex-situ* para incrementar sus poblaciones. Según el Libro Rojo español, la situación actual de *E. viviparum* sugiere que el método de cultivo *in vitro* podría ser un buen enfoque para preservar su germoplasma e implementar programas de reintroducción con las nuevas plantas obtenidas para reforzar su hábitat original. En el caso de especies en peligro de extinción, la mejor opción para iniciar el cultivo *in vitro* es utilizar semillas permitiendo así, preservar la diversidad genética de las plantas resultantes. Adicionalmente, si las especies estudiadas pueden tener problemas de germinación, los métodos de germinación *in vitro* permiten mejorar la tasa de germinación en comparación con los métodos tradicionales. Asimismo, si la especie de estudio tiene pocas semillas disponibles o su tasa de germinación es muy baja, algunos autores recomiendan la propagación de las plántulas obtenidas a través de la germinación por micropropagación. Esta técnica multiplicará las plántulas obtenidas durante la germinación permitiendo obtener un mayor número de nuevas plantas. Con este incremento de material vegetal se puede fortalecer las poblaciones de los hábitats afectados (programas de repoblación),

estudiar más a fondo la especie amenazada reduciendo la presión en su medio natural y / o preservar germoplasma *in situ* y colecciones vivas.

En cuanto a su etnobotánica, *E. viviparum* no tiene ningún uso conocido. Sin embargo, pertenece a un género y familia (Apiaceae) muy conocido y utilizado en la medicina popular como remedio para diferentes enfermedades. En los últimos años, los estudios sobre las especies más utilizadas de esta familia han mostrado una correlación entre sus usos medicinales y sus compuestos bioactivos. Esto aumenta el interés por el cultivo *in vitro* de esta especie, ya que este tipo de metodología presenta varias ventajas, en comparación a las plantas cultivadas o salvajes, para la obtención de estas moléculas bioactivas. Las plantas cultivadas a través de esta técnica tienen concentraciones más altas de los metabolitos de interés y su producción puede limitarse y / o aumentarse en diferentes tejidos vegetales. Además, la producción de compuestos se puede llevar a cabo durante todo el año independientemente de la estación. Por tanto, esta tesis aborda el estudio de la germinación y micropropagación *in vitro* de *E. viviparum*, para implementar una estrategia de conservación *ex situ* que contenga como punto innovador la valorización de la especie a través del estudio de sus compuestos fenólicos y actividades biológicas asociadas, que puedan ser de interés para futuras aplicaciones industriales. Así, la primera parte de este trabajo estudió la germinación de *E. viviparum* a partir del conocimiento recogido en trabajos preliminares sobre esta especie y otros sobre la familia y el género. Estos trabajos exponían que las semillas de esta especie presentan bajos porcentajes de germinación, gran cantidad de semillas inviables y otras viables que exhiben varios grados de dormición. Se realizaron varios experimentos donde las semillas de *E. viviparum* fueron sometidas a diversos factores previamente estudiados, capaces de eliminar la latencia que pudiera presentar esta especie, y posteriormente se incubaron en múltiples condiciones que podrían mejorar el nivel de germinación. Para un estudio completo se necesitarían numerosos experimentos que contengan todos los factores probados y así, obtener un espacio de diseño bien muestreado y amplio para estudiar en las mejores condiciones el efecto de cada variable. Sin embargo, una de las limitaciones de este trabajo es la baja cantidad de semillas que

se pueden recolectar en su hábitat, lo que restringe el espacio de diseño de la investigación. La estadística tradicional no consigue analizar bien estos resultados, sin embargo, a día de hoy existen herramientas de inteligencia artificial, como las redes neuronales (*Neurofuzzy Logic* en este caso), que analizan estos resultados con mayor precisión. Los resultados de este trabajo confirmaron la presencia de numerosas semillas no viables en *E. viviparum* (62,5%), una tasa similar a la de muchas especies de su género y familia. Estas semillas también presentaron la morfología típica exhibida por semillas no viables de su familia. Ambas morfologías carecen de embrión, pero mientras que unas tienen endospermo las otras están completamente vacías por dentro. Estas variaciones morfológicas se deben a que pueden ser infestadas por insectos (vacías) o pueden ser infértiles por su procedencia (autofecundación en la misma umbela). Otro resultado notable fue la evidencia de que las semillas de *E. viviparum*, al igual que muchas especies de su género, presentan un embrión subdesarrollado y, por tanto, dormición morfológica (DM). Para medir el efecto de los tratamientos utilizados sobre el desarrollo embrionario (necesario para germinar), utilizamos el ratio embrión: semilla (proporción entre la longitud del embrión y la semilla; ratio E:S). Esta proporción en las semillas de la población control (semillas sin tratamiento) fue de 0.31, similar a otras especies de la familia con DM. En cuanto al análisis estadístico, de los 9 factores iniciales Neurofuzzy Logic modeló los factores críticos y sus interacciones con alta predictibilidad y precisión (>75 R^2). El modelo permitió reducir el número de factores iniciales, de 9 a 6, que consideró los más críticos, por explicar un gran porcentaje de variación de los resultados obtenidos. Además, permitió identificar las mejores condiciones para el desarrollo embrionario de *E. viviparum* y mejorar las tasas de germinación de las semillas, una vez eliminadas las semillas no viables. Las condiciones óptimas son las descritas: un breve período de estratificación a 25ºC, seguido de incubación a 24ºC, durante 20 semanas en medio de germinación suplementado con giberelinas (fitohormona). Por tanto, los resultados obtenidos indican que las semillas de *E. viviparum,* además de tener MD, podrían tener un componente fisiológico adicional. La dormición fisiológica (DF) retrasa aún más su germinación y, en consecuencia, una parte de la población de

semillas puede presentar dormición morfofisiológica (DPM). Este componente fisiológico adicional debe ser eliminado para permitir el crecimiento del embrión y posteriormente, la germinación de la semilla. En los tratamientos más efectivos, la estratificación a 25ºC y posterior aplicación de giberelinas en el medio permitieron la rotura de este componente fisiológico. Esta eliminación permitió el crecimiento del embrión y la germinación después de la incubación a 24ºC, con un requisito de humedad, proporcionado por el medio de cultivo.

La segunda parte de esta tesis tuvo como objetivo incrementar el rendimiento de las plantas germinadas de la fase anterior mediante micropropagación. Asimismo, se realizó una determinación preliminar de los compuestos fenólicos totales y flavonoides, así como su actividad antioxidante relacionada mediante técnicas colorimétricas en las plantas obtenidas durante el cultivo *in vitro*. El protocolo de micropropagación utilizado se llevó a cabo siguiendo las fases descritas en la bibliografía. En primer lugar, las plántulas germinadas se mantuvieron en un medio de establecimiento (medio Murashige & Skoog) para asegurar que crecían sin contaminación interna y se desarrollaban con normalidad dentro del medio. Las plantas establecidas, durante la fase de multiplicación, se introdujeron en un medio suplementado con diferentes concentraciones de reguladores del crecimiento y se midió el efecto de estos sobre la formación de nuevos brotes. Los medios utilizados estaban suplementados con una combinación de dos citoquininas, 6-bencilaminopurina o BAP y kinetina o KIN, a 3 concentraciones: 0, 1 y 2 mg L^{-1}. Las plantas se trasfirieron cada 5 semanas a un nuevo medio (subcultivo) y en el quinto subcultivo, se comenzó un experimento paralelo para estudiar el alargamiento y enraizamiento de los brotes obtenidos. Durante este ensayo, se midió el impacto de la fuerza iónica del medio y el nivel de sacarosa (fuente de carbono) en el desarrollo y elongación de los brotes y raíces. Una vez enraizadas, las plantas se trasplantaron a macetas con turba y perlita, donde fueron aclimatadas a través de la reducción controlada de la humedad, que permite que las raíces y estomas de la planta proveniente del cultivo *in vitro,* vuelvan a ser funcionales. Durante la fase final de este ensayo se utilizaron los brotes formados durante la multiplicación para extraer los compuestos fenólicos. El extracto

resultante se utilizó para determinar: el contenido de compuestos fenólicos totales mediante el método de *Folin Ciocalteu*; el contenido total de flavonoides por el método colorimétrico de cloruro de aluminio, y la actividad antioxidante por el método DPPH (1,1-difenil-2-picrilhidrazilo). Respecto a los resultados obtenidos, en la fase de establecimiento, las plántulas provenientes de la germinación, sobrevivieron y se desarrollaron sin presentar contaminación. En la fase de multiplicación, los medios suplementados con 2 mg L^{-1} de BAP originaron el mayor número de nuevos brotes. Asimismo, la combinación con la KIN aumentó significativamente el número de nuevos brotes en comparación a los tratamientos que solo presentaban una citoquinina. Los medios suplementados con 2 mg L^{-1} BAP en combinación con 1 o 2 mg L^{-1} de KIN obtuvieron los mejores resultados con 5.1 y 5.8 nuevos brotes por plántula. En la etapa de elongación de brotes y enraizamiento, el medio de cultivo con la mitad de fuerza iónica y 2 % de sacarosa, fue el que mejores resultados tuvo en todos los parámetros medidos: alargamiento de brotes, longitud de raíz y peso seco de raíz. Finalmente, en la etapa de aclimatación, el 96% de las plantas trasplantadas sobrevivieron y fueron trasladadas a su hábitat natural. Con respecto a la determinación preliminar de compuestos fenólicos y actividad antioxidante, los extractos tuvieron una actividad antioxidante moderada en comparación con otras especies del género. Además, el incremento de esta actividad se correlacionó significativamente con la concentración de compuestos fenólicos, que aumentaron significativamente con niveles más altos de BAP. Por lo tanto, gracias a este ensayo establecimos un protocolo de micropropagación que puede ser utilizado como estrategia de conservación *ex-situ* de *E. viviparum*. Además, la especie mostro potencial fitoquímico y es de gran interés estudiar en profundidad sus compuestos fenólicos y las bioactividades relacionadas.

Así, la última parte de este trabajo evaluó la composición fenólica de las partes aéreas y raíces de plantas de *E. viviparum* provenientes de cultivo *in vitro*. Asimismo, se evaluaron las actividades biológicas antioxidantes, citotóxicas y antimicrobianas (antibacterianas y antifúngicas). Para estas determinaciones, las

muestras aéreas y radiculares del medio MS sin citoquininas se sometieron a una extracción sólido-líquido en una solución hidroetanólica (80:20; v/v) que fue utilizada para las posteriores determinaciones. La determinación de compuestos fenólicos se realizó mediante HPLC-DAD-ESI / MSn. La actividad antioxidante se determinó mediante dos ensayos específicos basados en células. El primer ensayo antioxidante se realizó a través del método de sustancias reactivas al ácido tiobarbitúrico (TBARS). Este método mide la concentración a la que los extractos pueden prevenir la formación de malondialdehído (MDA) a través de la donación de átomos de hidrógeno a las especies radicales peroxilo formadas durante la oxidación lipídica en las células (en este ensayo, células de cerebro de cerdo). El segundo ensayo utilizado fue el método de inhibición del hemólisis oxidativa (OxHLIA). Este ensayo establece el tiempo y la concentración a la que los antioxidantes pueden retrasar la hemólisis de los eritrocitos utilizados, al capturar los radicales hidrófilos y / o lipófilos en las membranas de eritrocitos de ovejas. La actividad citotóxica se determinó mediante el método de sulforrodamina B en cuatro líneas de células tumorales, NCI-H460 (carcinoma pulmonar de células no pequeñas), MCF-7 (adenocarcinoma de mama), HeLa (carcinoma cervical) y HepG2 (carcinoma hepatocelular). Los extractos también se probaron para determinar su actividad hepatotóxica en una línea celular de hígado porcino no tumoral (PLP2). Finalmente, se utilizó el método de microdilución para determinar la actividad antibacteriana utilizando varias bacterias Gram-positivas y Gram-negativas, y la actividad antifúngica para los hongos Micromycetes.

Durante la determinación de compuestos fenólicos se identificaron 14 compuestos de las partes aéreas y raíces cultivadas *in vitro* de *E. viviparum*. Diez de estos compuestos fueron ácidos fenólicos y 4 flavonoides, siendo los primeros los principales compuestos fenólicos en ambos órganos (38,3 mg g^{-1} en las partes aéreas y 102 mg g^{-1} en las raíces). El principal compuesto cuantificado fue el ácido *trans* rosmarínico, que representó más del 70 % de todos los compuestos fenólicos identificados, en ambos órganos. Este compuesto es conocido por su alta capacidad antioxidante, antimicrobiana y anticancerígena, así como por sus efectos neuro y / o cardioprotectores. Aunque se identificaron flavonoides en ambos órganos, solo

se cuantificaron en la parte aérea, siendo la tectorigenina -*O*- glucurónido, el flavonoide mayoritario identificado. En cuanto a las determinaciones de la actividad antioxidante, los resultados de OxHLIA mostraron la concentración de extracto necesaria para proteger la hemolisis del 50 % de la población de eritrocitos. Dado que los extractos contienen diferentes moléculas que ofrecen protección en diferentes períodos, los valores se midieron para Δt de 30 y 60 minutos. Los extractos de *E. viviparum* mostraron una protección moderada en comparación con el antioxidante sintético utilizado como control, Trolox, siendo el extracto de raíz el más eficaz para ambos intervalos. En cuanto al ensayo TBARS, los resultados revelaron la concentración de extracto necesaria para inhibir la formación de especies reactivas producidas durante el proceso de peroxidación lipídica. La capacidad antioxidante en este ensayo también fue mayor para el extracto de raíz, pero menos efectiva que Trolox.

Las plantas son esenciales para muchos procesos vitales en la tierra, sin embargo, su tasa de extinción ha mostrado un aumento exponencial, en los últimos años, nunca antes estudiado. Además de los procesos de vida que llevan a cabo, las plantas son fuente de múltiples metabolitos minoritarios que se utilizan para su interacción con otras plantas y con el medio que las rodea. Estos compuestos, conocidos como metabolitos secundarios, también están asociados con múltiples actividades biológicas deseables para los seres humanos. Así, las plantas que contienen estos metabolitos se han utilizado durante décadas para el bienestar humano a través de la medicina tradicional y hoy en día para muchas aplicaciones de interés en diversas industrias. Por tanto, la acción coordinada para la conservación de las especies vegetales parece importante, no solo por sus procesos vitales de los que podemos beneficiarnos, sino también, porque podrían ser rentables como fuentes productivas de compuestos ya conocidos o para el descubrimiento de nuevos que puedan ser explotados. En el caso de *E. viviparum,* su actividad antioxidante muestra que el extracto de raíz en ambas pruebas podría estar correlacionada con los compuestos del ácido cafeico y rosmarínico, que tienen una alta capacidad antioxidante y fueron encontrados en altas

concentraciones en este órgano. Con respecto a los resultados de citotoxicidad, estos mostraron que las células del carcinoma hepatocelular (HepG2) eran las más susceptibles a los extractos de *E. viviparum* y que se requería una concentración baja para inhibir el 50% de su crecimiento sin presentar toxicidad, en el rango de concentraciones ensayadas, en las células no cancerígenas del hígado (PLP2). Sin embargo, los extractos no fueron efectivos contra las células de carcinoma cervical (HeLa). Finalmente, durante los ensayos antimicrobianos, las bacterias gram negativas fueron las más sensibles a los extractos probados. Por un lado, el extracto de raíz fue más eficaz contra las cepas de *Bacillus cereus* y *E. coli*, aisladas de alimentos y ATCC 25922, respectivamente. Asimismo, el extracto de parte aérea fue muy efectivo contra *Salmonella typhimurium*, presentando una menor inhibición y concentraciones bactericidas en comparación con el control positivo ampicilina. El extracto de raíz fue más eficaz que el ketoconazol (control antifúngico) en la inhibición del crecimiento y la eliminación de colonias de *Penicillium ochrochloron* (ATCC 9112). Por último, *P. funiculosum* y *P. verrucosum* var. *cyclopium* fueron las cepas de hongos más susceptibles a ambos extractos, requiriendo una menor concentración de extracto para inhibir el crecimiento y matarlos en comparación con las otras colonias testadas. Por todo ello, la última parte de la presente tesis caracterizó a esta especie *in vitro,* como una interesante fuente de compuestos fenólicos, particularmente sus raíces, que contienen ácido *trans* rosmarínico y ácido *trans* 3-*O*-cafeoilquínico en alta concentración. Del mismo modo, el extracto de raíz también presentó alta actividad antioxidante, citotoxicidad moderada en las diferentes líneas de células tumorales probadas y ninguna hepatotoxicidad en células de hígado porcino no tumorales (PLP2). Además, también mostró mejores resultados en actividad antimicrobiana que el extracto de las partes aéreas, siendo más efectivo contra *P. ochrochloron* que los controles positivos utilizados durante este ensayo. En conjunto, este estudio destaca el interés en la conservación de esta especie debido a la presencia de diferentes compuestos fenólicos de interés, que aportan propiedades antioxidantes y antimicrobianas que pueden ser de utilidad para diversas bio-industrias.

Así, esta tesis describe con éxito, por primera vez, una estrategia integrada para la conservación *ex situ* de *Eryngium viviparum* mediante procedimientos de germinación y cultivo *in vitro*, añadiendo un nuevo enfoque, a través de la investigación de posibles metabolitos secundarios con diversas actividades biológicas que agreguen un valor extra a la especie de estudio y a su proceso de conservación.

Introduction

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General Introduction

Plant threatened species: a real problem

Global biodiversity drops at an unprecedented rate, and the number of endangered species exceeds the available conservation resources (Myers et al. 2000; Tilman et al. 2017). Plants are vital for life on earth since they transform the energy of the sun into oxygen through photosynthesis. In addition to their essential function, plants have also been used for multiple industrial purposes during the past centuries, such as food, dyes, poisons, stimulants, insecticides, and flavours, mainly due to their secondary metabolites properties (Li and Vederas 2009; Aharoni and Galili 2011; Atanasov et al. 2015). However, they are the organisms with the highest biodiversity loss and, according to the IUCN Red List of Threatened Species, from a total of 38630 plants described as threatened, 29254 were added in the last ten years. In Europe, Spain has one of the highest numbers of endemic threatened plants, from which 1315 are already listed in the red book (IUCN Resources 2020).

Humans are entirely dependent on Earth's ecosystems; hence, biodiversity losses will result in a decrease in their services for human well-being. Few studies emphasise that despite the challenge to recover endangered species, a scientific understanding of threats and their impacts enables the design of an effective conservation strategy (Sarasan et al. 2006; Monks et al. 2019). The conservation actions to assess the decline and enhance recovery will avoid both extinction and the loss of potential new sources for human uses. The management of wild populations in their natural habitat is the most effective conservation strategy and is known as *in situ* conservation. This traditional strategy attempts to conserve plant species through the protection of their habitat, allowing the preservation of plant interactions with their ecosystem (Volis and Blecher 2010; Oldfield et al. 2019). In Spain, *in situ* strategies have been implemented for several plant species. However, threats as overgrazing, territory pressure, and competition triggers the reduction, fragmentation, and degradation of these plant species and their natural habitats, even those growing in protected zones (González-Benito and Martín 2011). In these cases, *in situ* conservation needs to be complemented by *ex situ* approaches,

which involves the storage of heterogeneous genetic samples of threatened species outside their natural environment. (Cohen et al. 1991; Glowka et al. 1994; Sarasan et al. 2006; González-Benito and Martín 2011). Botanical gardens usually are responsible for the conservation of germplasm through the creation of living collections of plants, seed bank for long term storage, and sometimes the application of cell or tissue *in vitro* cultures, and cryopreservation techniques. Seed bank is the most utilised strategy as these facilities have low cost maintenance, protect seeds from predation or infestation, and allow the storage of a significant number of seeds during extended periods with minimal risk of genetic damage (Edwards and Jackson 2019; Faraji and Karimi 2020). However, some species have a low germination rate or recalcitrant seeds although maintaining their vegetative propagation. Therefore, in these cases, *in vitro* culture is an efficient method to increase the number of individuals and use the produced specimens to reintroduce them into their natural environment through specialised programs (Grigoriadou et al. 2019; Streczynski et al. 2019). Additionally, the plant material obtained through *in vitro* culture allows a more in depth study of the selected threatened species without the need to collect and reduce their number in the original habitat (Hulme 2011; Engelmann 2011).

Eryngium viviparum: current situation

Eryngium viviparum (J. Gay, 1848) is a small perennial plant (2–10 cm) with basal leaves that form a rosette. The characteristic rosette is composed of lanceolate leaves with parallel veins, pinnatifid, narrow petioles, and thorny margins (Fig 1A). It is a hydrophyte plant belonging to the Apiaceae (Umbelliferae) family and Saniculoideae subfamily, which lives in wetlands distributed in Spain, Portugal and France. Their natural habitat is characterised by a flat area subjected to seasonal flooding, living submerged for 7–9 months of the year (Castroviejo et al. 2003; Bañares et al. 2004; Serrano et al. 2019). The morphology of *E. viviparum* presents significant differences between the flooded and the dried period. The basal rosette formation is persistent all year. However, when the bloom begins, during the dry period, they develop dichotomous-branching flower stems (stolon that grows at the soil surface level), which possess small opposed acute lanceolate leaves (Fig 1B).

The flowers are grouped in small globose capitulum-type inflorescences, and the fruits are schizocarps, which upon maturation separates into two seeds or mericarps (Fig 1C and 1D; Rascle, 2018; Rascle et al., 2018).

Fig 1. *Eryngium viviparum*: A) Picture taken by Christophe Girod from Jardim Botânico UTAD during the dried period, Flora Digital de Portugal. B) Draw of basal rosette, dichotomous-branching flower stem, and fruit (Danton and Baffray 1995). C) Detailed schizocarp fruit with two seeds or mericarps. D) Detail of a seed or mericarp.

E. viviparum was classified as *vulnerable* by the International Union for Conservation of Nature (IUCN) and included on its Red List of Threatened Species since 1997 (Walter and Gillett 1998). However, its condition worsened to *endangered* due to its population decreased to less than 80 km^2, losing many subpopulations and increasing the remaining populations fragmentation (Lansdown 2011; IUCN Resources 2020).

In France, it was considered a well-established plant in the '80s, with more than 40 populations identified in the country (Rascle 2018; Rascle et al. 2018). However, nowadays, the only known community alive is based at the Belz's natural reserve and belongs to the Morbihan department at the Brittany province, in north-western of France. The plant is now considered *critically endangered* in France, and a National Action Plan coordinated by the national botanical conservatory of Brest was developed for its conservation (Magnanon et al. 2012; Rascle et al. 2019). Although several management efforts have been made in this population, allowing an increase in the number of individuals; the re-establishment of the surrounding extinct populations seems essential for long-term conservation. A restoration

program attempted to use several sites which have a historical presence of this species, adapting the habitat conditions for a spontaneous appearance of the plant from the soil seed bank, without success. Therefore, the reintroduction to generate new populations has become the only possible solution in this area to mitigate the negative consequences of isolation and to recover the genetic diversity of this species. Experimental reintroduction of both germinated plants and seed was carried out in 2015. The transplantation of new individuals to these zones presented some degree of success; however, seed sowing was not sufficient for the establishment of new populations (Rascle et al. 2018).

In Portugal, the three identified *E. viviparum* communities nearby Porto were extinct due to the city expansion. However, when this species was thought to no longer be present in Portuguese territory, a population was found in the north-east corner, in Nogueira, one of the three mountain ranges of the region. The existence of this population is natural, since this area is located between two identified communities in Spain, Ourense and Zamora. It is restricted to a single pond near the road and can be easily destroyed by any forest management activity. To the author best knowledge, no conservation actions have been implemented in Portugal to preserve this population (Aguiar 2003).

Finally, in Spain, 36 small areas have been identified and described in the last report regarding this species, which can be distributed in five main populations (Romero et al. 2004). In Galicia, three populations were known; however, the one in Melide (A Coruña) was extinct, being the two living communities located in Terra Chá (Lugo) and Limia (Ourense). In Castilla y León, *E. viviparum* is present in several provinces: Zamora, with broader distribution through the basin of Tera river and Sanabria lake; León, in Páramo Leonés region; and Palencia, in Mantinos locality (del Egido et al. 2020). The number of individuals in these provinces exhibit extreme fluctuations each year, specifically population from Lugo, which presented a regression trend (Romero et al. 2004).

The Galician *E. viviparum* population's threats are similar to those presented by foreigner populations, the changes in land usage, water drainage, and

constructions have dictated the loss or decline of these communities (Berastegi 2016). Concerning Castilla y León, grazing and water pollution are the main threats; being their communities less threatened than specimens from Galician. Moreover, the tendency to have less rainfall due to climate change is another general threat factor for the plants which live in the wetlands (Lefebvre et al. 2019). Several *in situ* conservation measures, through the LIFE + Tremedal project, have been carried out in these populations. The protection and restriction access to the site favoured the flooding through topographic corrections for the modelling of the land, and the eradication of exotic and invasive species (Bañares et al. 2004; Berastegi 2016). Additionally, some populations are located in protected areas, such as SIC ("Sites of Community Importance" or LIC "Lugares de Importancia Comunitaria") or SAC ("Special Areas of Conservation" or ZECs "Zonas de Especial Conservación") (Serrano et al. 2019)

Despite these policies, the plant status remains critical, and different *ex situ* conservation strategies have been suggested for this and other species in the same situation within the Iberian Peninsula. Micropropagation was one of the techniques recommended for *E. viviparum* conservation since this method quickly increases the number of individuals, to obtain new plant material with high-value for living communities for reintroduction, research, and the reduction on the pressure on existing natural populations (González-Benito and Martín 2011).

Main strategies for *Erygium viviparum* conservation

Seeds as source of plant material for conservation

Seeds are the preferred vegetative material source for conservation strategies involving a propagation process since they maintain a broad genetic base in the generated new plants (Cruz-Cruz et al. 2013). *In vitro* methodologies may increase the seed germination rate comparing to conventional methods, especially in the cases where dormancy or other particular seed requirements cause limitations into the process. Moreover, after germination, if few seeds were available, might be necessary an additional *in vitro* proliferation step to obtain new seedlings (Fay 1992; Engelmann 2011).

Information found in the literature about the *E. viviparum* seed germination process is still scarce (Rascle et al. 2019). Regarding their sexual reproduction, the bloom occurs between July and September. One basal rosette could produce many hermaphrodites' flowers grouped in inflorescences. Although *E. viviparum* fecundation has not been properly studied, from the available data seems to be an allogamous process (cross-fertilization), with pollen viability higher than 80%. Its fruits are schizocarps, each containing two mericarps or seeds (Buord et al. 1999). Field observation indicates that *E. viviparum* seeds usually reach maturity in the early summer season; nevertheless, due to annual season oscillations, the maturation process might be extended until the end of the summer season. Depending on the moment seeds reach maturation, they might germinate in the early autumn or the next spring season. This change in seed maturation time also represents a decrease up to 20-30% in the viability of mature seeds (Buord et al. 1999; Rascle 2018). In addition to this problem, preliminary germination tests of seeds collected in France and Spain carried out at the Brest´s National Botanical Conservatory showed strong dormancy together with non-uniform and highly variable rates, which prevented germination up to 40% (Perrin and Magnanon 2007).

Since there is scarce information available regarding *E. viviparum* germination, data from other plants from its family and genus could be helpful to fill the literature gap and promote a better discussion and understanding regarding the new findings. The Apiaceae family and the *Eryngium* genus exhibit poor germination rates (Baskin and Baskin 2004; Mozumder et al. 2011; Thiem et al. 2013; Rascle et al. 2018). The family seeds are characterised by containing underdeveloped embryos, which are defined as morphologically dormant seeds due to a small delay in the germination process. This dormancy process requires suitable environmental conditions to develop the embryo before seed germination (Martin 1946; Vandelook et al. 2007, 2008). However, in suitable conditions, not all the seeds achieve germination due to the presence of an additional physiological mechanism that inhibits embryo development and must be broken before germination (Baskin and Baskin 2004; Vandelook et al. 2009). Moreover, Apiaceae

family may present non-viable seeds, fully empty or without embryos. Seed viability depends totally on the presence of the embryo; embryoless seeds may appear due to different causes, including degeneration of the zygote, failure on embryo development by mutation, infertile hybrids, and insect predation (Robinson 1954; Baskin and Baskin 2014). Although, dormancy and germination are interconnected; they are generally studied independently. Dormancy-breaking is a process required for germination to occur; it is almost impossible to define the beginning and the end of each step, mainly due to interaction or counteraction factors in both processes. Therefore, to fully understand both processes, it is necessary to integrate multidimensional data to describe the complex biological interactions instead of traditional statistical methods, which cannot be fully understood through a simple comparison of means among treatments, analysis of variance, regression models or simple algorithms (Struik et al. 2005; Landín et al. 2009; Gallego et al. 2011). Artificial intelligence (AI) tools have been useful techniques for establishing relationships between multiple variables (factors and parameters). Furthermore, several studies have shown the effectiveness of those AI tools for modelling and optimizing complex biological processes, such as germination and dormancy (Cartwright 2008; Gago et al. 2010b, a; Niazian and Niedbala 2020). Based on the available data regarding this threaten species, it seems evident that, for an effective conservation strategy, it is necessary to investigate and establish a protocol to promote *E. viviparum* germination.

Micropropagation as source of plant material for conservation

E. viviparum can reproduce asexually through clonal fragmentation. During the water shortage periods, late spring and summer, this species possesses the remarkable capacity of vegetative propagation. Thus, *E. viviparum* reproduction process can occur from basal rosettes at the bloom period through two different routes (Fig. 2): i) propagules develop from the floral stolon during the flowering stage and ii) adventitious rosettes from the mother rosette at the root level. In both cases, newly formed basal rosette are obtained (Fig 2; Rascle et al., 2018).

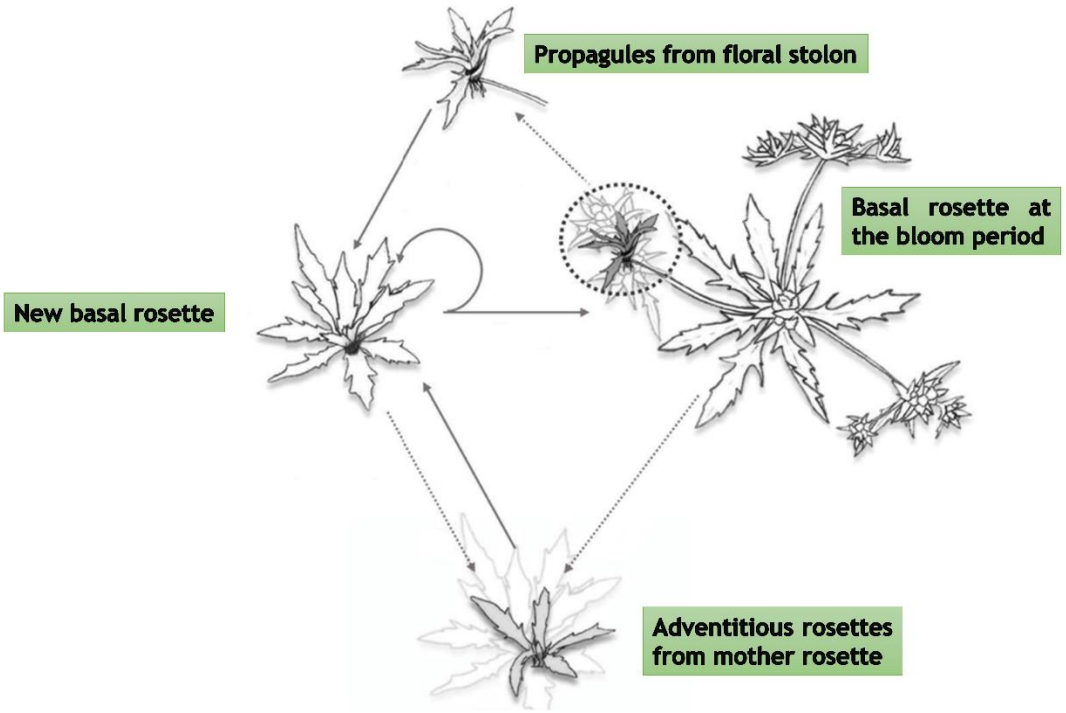

Fig. 2. *Eryngium viviparum* vegetative multiplication patterns. Modified from Rascle (2018).

Micropropagation was suggested as an *in vitro* culture technique for *ex situ* conservation of *E. viviparum* (González-Benito and Martín 2011). This method aims to multiply the number of plant individuals, via Plant Tissue Culture (PTC) technology, to obtain microplants true-to-type (clones). The micropropagation process can be initiated from the following three sources:

- Plant meristematic tissues that have a pre-existing apical or axillar meristems, which can grow and proliferate directly.
- Plant differentiated tissues that do not have meristem and consequently, it is necessary to induce adventitious organogenesis with plant growth regulators. It can be initiated directly, through tissue cultures from the mother plant, or indirectly from unorganized cells (callus).
- Embryos, either from natural seeds (zygotic embryos) or those adventitious (somatic embryos) which can develop new plantlets.

According to (George et al. 2008), the micropropagation procedure is divided into several stages:

- **Stage 0. Mother plant selection:** Select a representative stock of the plant species to be studied, free from disease symptoms. In the plant conservation field, it is preferred to avoid methods that may induce somaclonal variation, such as indirect organogenesis and/or somatic embryogenesis (Fay 1992; González-Benito et al. 2020). Thus, in the present doctoral work, seedlings with pre-existing meristems were used to begin the culture.

- **Stage 1. Culture establishment**: Obtaining an aseptic culture accustomed to growing in the culture media. This stage finishes when a number of suitable plant explants can survive without contamination, allowing the explants to continue to the next stage.

- **Stage 2. Multiplication:** The process to be carried out in this stage depends on the initial selected plant material. In tissues with pre-existing meristems (apical shoot tip and/or axillar buds), the new formed shoots are obtained for direct organogenesis (multiplication), while in differentiated tissues (without meristems), new formed adventitious shoots can emerge directly (direct organogenesis) or from callus (indirect organogenesis). During this stage, the different propagules formed can be divided and cultured again in cycles of multiplication (subcultures), allowing an increase in their number. Phytohormones or plant growth regulators (PGR) are essential for successful propagules formation, being cytokinins, auxins, and their interaction, the most important factors to regulate shoot multiplication and development into tissue cultures. Reports on different *Eryngium* species cultured *in vitro* indicate the formation of shoot rosettes during this stage (Thiem et al. 2013; Kikowska et al. 2014). This formation tends to produce shoot clusters hard to individually separate, which is a necessary step for their successful use in subcultures or transference to the natural environment.

- **Stage 3. Rooting:** Cytokinins used in stage 2 remove the shoots apical dominance, promoting the formation of new axillary shoots. Consequently, shoots from this stage are usually small, rootless, and non-functional when removed from culture media. Therefore, during stage 3, preparation of the shoots for the natural environment, it is necessary to induce root rhizogenesis in some cases, it requires

an additional step for full shoot elongation. In the present work, the formed shoot clusters needed the elongation step to assure successful transference to the soil (*ex vitro*).

Stage 4: Acclimatization: This stage needs a careful execution to avoid any loss of the *in vitro* propagated material. The rooted *in vitro* plantlets are mixotrophic, which means that they unable of produce their own requirements for health development (autotrophy), and are still fully dependent on the carbohydrates added to the medium. Moreover, and due to the high humidity exposure during plant growth in culture media, their stomata are also unable to close properly when are exposed to low humidity environments. Therefore, for successful acclimatization, and to overcome the particularities mentioned above, plantlets require to recover the usual stomata functionality and be able to persist under different natural environmental conditions; plantlets should be submitted to temperature oscillations and successive humidity reduction every week, over a month. The acclimatization stage can be carried out *in vitro* or *ex vitro*, in the last case, suitable substrates (e.g. peat and perlite) are generally used to facilitate an efficient root development (Gago et al. 2010a).

Therefore, micropropagation technique enables mass endangered plant propagation, implementing new ways of regeneration capacity, and as germplasm storage, thus preserving a representative genetic diversity, which constitutes a reliable methodology for plant conservation. Furthermore, the high number of plants produced, via micropropagation, would allow the establishment of plant reintroduction programs and a continuous supply of plant material for other purposes, such as the study of their bioactivities and related compounds.

Eryngium valorisation as source of bioactive compounds

Plant secondary metabolites are a diverse group of compounds biosynthesised in low quantities through different biochemical pathways, which have multiples functions in the life cycle of plants. Mostly, they are the mediators between plant-plant and plant-environment interactions, part of the plants protection system, and plant reproduction promotors through the attraction of pollinators (Bourgaud et

al. 2001; Verpoorte and Memelink 2002). A large number of biological activities have also been described as associated whit these metabolites, giving them high-value as compounds with potential for different industrial applications in food, pharmaceutical, cosmetic, or agrochemical markets (Jacobo-Velázquez and Cisneros-Zevallos 2012).

To the author best knowledge, applications for secondary metabolites of *E. viviparum* are not currently known. However, aerial and root parts from other *Eryngium* species have been used in traditional medicine as a treatment for several diseases: skin, kidney, infections, tumours, hypertension, among others. Recently, the phytochemical potential of this genus have been confirmed through the discovery of many bioactive compounds, mainly phenolic and terpenes, which have shown different biological activities in both *in vitro* and *in vivo* assays (Wang et al. 2012; Vukic et al. 2018). Among those bioactivities: cytotoxic (in cancer cell lines of the colon, prostate, pancreas, lung, and leukaemia); anti-inflammatory; antibiotic, anti-fungal, anti-parasites; and antioxidant have been described (Lisciani et al. 1984; Küpeli et al. 2006; Wang et al. 2012; Singh et al. 2013; Erdem et al. 2015). Therefore, the presence of some secondary metabolites, mainly phenolic and terpenes compounds, in the *Eryngium* genus outlines their potential use in several industrial applications (Kikowska et al. 2012; Thiem et al. 2013).

Phenolic compounds are the natural protection substances widely distributed in the plant kingdom and their concentration is influenced by genetic, environmental, physiological, and geographic factors (Tungmunnithum et al. 2018). Plant phenolic compounds include phenolic acids, flavonoids, tannins, and to a lesser extent, stilbenes and lignans (Dai and Mumper 2010). These compounds have been identified as antioxidants with high potential to prevent various oxidative stress-associated diseases (García-Pérez et al. 2018). Additionally, polyphenols have other specific bioactivities related to protein-interactions in different biological pathways, which may have a potentially positive effect on the treatment and prevention of some diseases. Several *in vitro* and *in vivo* assays have demonstrated their effectiveness in cardiovascular, neurodegenerative, and cancer diseases, as well as their ability to modulate some enzymes and cell receptors (Arts

and Hollman, 2005; Cole et al., 2005; Rasmussen et al., 2005; García-Pérez et al., 2019).

Moreover, plant *in vitro* culture techniques not only allow to obtain a higher amount of plants but also a controlled increase in the production of their bioactive compounds, with total independence of geo-climatic limitations (Atanasov et al. 2015). Furthermore, it is a safe and predictable method to isolate specific compounds by increasing the distribution of the desired molecules in the different plant tissues. Additionally, *in vitro* culture can modulate the plant growth rate and their metabolite yields in several-fold through targeted elicitation (Matkowski 2008; Dias et al. 2016; Gonçalves and Romano 2018).

Thus*, in vitro* culture of threatened plants with medicinal potential is a promising approach to ensure their conservation and promote an extra added-value through their metabolites identification and bioactivities determination.

Objectives

Objectives

The present Ph. D. thesis was aimed to explore new ways for the conservation of *Eryngium viviparum* species. Thus, we hypothesised that "*Eryngium viviparum* could be micropropagated from seeds, opening a new strategy for its *ex situ* conservation without damaging its natural populations, and producing enough *in vitro* plant material for its reintroduction and evaluation of its phytochemical and therapeutically potential".

To assess that hypothesis, the following specific objectives were proposed:

1.- Decipher the mechanism of seed dormancy of *Eryngium viviparum* seeds by artificial intelligence algorithms.

2.- Establish a new *ex situ* conservation strategy through micropropagation procedure and the evaluation of phytochemical potential of *Eryngium viviparum.*

3.- Determination of *Eryngium viviparum* phenolic compounds and their associated bioactivities.

Results

Computer-Assisted recovery of threatened plants: keys for breaking seed dormancy of *Eryngium viviparum*

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Computer-Assisted Recovery of Threatened Plants: Keys for Breaking Seed Dormancy of *Eryngium viviparum*

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Many endangered plants such as *Eryngium viviparum* (Apiaceae) present a poor germination rate. This fact could be due to intrinsic and extrinsic seed variability influencing germination and dormancy of seeds. The objective of this study is to better understand the physiological mechanism of seed latency and, through artificial intelligence models, to determine the factors that stimulate germination rates of *E. viviparum* seeds. This description could be essential to prevent the disappearance of endangered plants. Germination *in vitro* was carried out under different dormancy breaking and incubation procedures. Percentages of germination, viability and E:S ratio were calculated and seeds were dissected at the end of each assay to describe embryo development. The database obtained was modeled using neurofuzzy logic technology. We have found that the most of *Eryngium* seeds (62.6%) were non-viable seeds (fully empty or without embryos). Excluding those, we have established the germination conditions to break seed dormancy that allow obtaining a real germination rate of 100%. Advantageously, the best conditions pointed out by neurofuzzy logic model for embryo growth were the combination of 1 mg L^{-1} GA_3 (Gibberellic Acid) and high incubation temperature and for germination the combination of long incubation and short warm stratification periods. Our results suggest that *E. viviparum seeds* present morphophysiological dormancy, which reduce the rate of germination. The knowledge provided by the neurofuzzy logic model makes possible not just break the physiological component of dormancy, but stimulate the embryo development increasing the rate of germination. Undoubtedly, the strategy developed in this work can be useful to recover other endangered plants by improving their germination rate and uniformity favoring their *ex vitro* conservation.

Keywords: Apiaceae, artificial intelligence, conservation, germination, dormancy, endemic plant, embryo seed ratio, underdeveloped embryo

INTRODUCTION

Eryngium viviparum is a small biennial aquatic plant that belongs to Apiaceae family, endemic to the European Atlantic region and with distribution in NW France, NW Portugal and NW Spain (Romero et al., 2004). Their natural habitat is flat areas subjected to seasonal flooding, living submerged for 7–9 months of the year. Aquatic plants are one of the most threatened groups in the European flora, mainly due to anthropic habitat alteration and destruction (Romero et al., 2004; Ramil-Rego and Dominguez-Conde, 2006; Magnanon et al., 2012). In 1997, *E. viviparum* was classified as *vulnerable* by International Union for Conservation of Nature (IUCN) and included in red list of threatened plants (Walter and Gillett, 1998). Moreover, *E. viviparum* has been listed in Annex I of the Berne Convention and considered by Directive 92/43/EEC as a priority species (Annex II). Its classification has recently been changed to *endangered* due to the loss of many subpopulations, the drastic reduction of the area of occupancy (<80 km^2 in the world) and the decrease in the quality of its habitat and of the number of individuals (Lansdown, 2011).

The main threats for endangered plants are human pressure over the territory, competition with other species, overgrazing and collecting that cause degradation, fragmentation, and massive reduction of their natural habitats (Ramil-Rego and Dominguez-Conde, 2006; González-Benito and Martín, 2011; Lansdown, 2011; Magnanon et al., 2012). Although high protection and access restrictions to their natural habitats have been implemented, for *in situ* conservation, the situation is critical for some species as *E. viviparum*. New *ex situ* conservation strategies, such as *in vitro* propagation are needed and should be urgently designed and implemented in order to protect them. Different micropropagation procedures have been developed for endangered and endemic plants in the past 30 years and, also for this particular species (Ba-ares et al., 2004; González-Benito and Martín, 2011). The critical population size of *E. viviparum* does not allow sufficient material from its natural habitat for micropropagation. Therefore, Fay (1992) has proposed its seeds as the material of choice for *ex situ* conservation, which allows preserving their biodiversity.

Many species of Apiaceae family are well known by a non-uniform and asynchronized seed germination (Mozumder and Hossain, 2013), which promotes germination rates lower than 10% and limits their *in situ* conservation. Moreover, low germination in Apiaceae is due to several kind of dormancy state in seeds and by the presence of high amount of non-viable seeds (Robinson, 1954; Ojala, 1985). Different strategies have been developed to overcome dormancy problems in several Apiaceae, including cold and/or warm stratification (Necajeva and Ievinsh, 2013), the use of plant growth regulators such as gibberellic acid (GA_3) and kinetin (KIN) (Mozumder and Hossain, 2013) or the incubation temperature (Finch-Savage and Leubner-Metzger, 2006). As far as we know no studies have addressed the low germination rate problem in *E. viviparum,* which remains a challenge.

In recent years, several artificial intelligence tools have been applied to model and predict the effect of different variables in plant tissue culture (Zielinska and Kepczynska, 2013), monitoring seed growth and vigor (Chaugule, 2012) or testing germination (Dell'Aquila, 2004). In this work, neurofuzzy logic has been applied to investigate the cause-effect relationships between several germination factors (dormancy breaking stratification and germination conditions) and seed germination responses (percentage of germination, embryo seed rate, etc.). Neurofuzzy logic is a hybrid approach that combines the adaptive learning capabilities from artificial neural networks with the generality of representation from fuzzy logic through simple "IF-THEN" rules. This methodology has been previously and successfully used as advanced decision support tool (Gallego et al., 2011; Gago et al., 2014).

On this purpose, this study attempts to get insight on the germination of *E. viviparum* seeds and the causes of its low germination rate. Neurofuzzy logic is applied to model germination results as a function of several germination conditions in order to find the key factors that control or stimulate the embryo development, facilitating the completion of germination. Understanding the physiological mechanism responsible for seed germination should help to design new procedures for any other endangered plant with low germination rates.

MATERIALS AND METHODS

Plant Material

Fruits (schizocarps) of *E. viviparum* Gay were collected on the margin of the Cospeito Lake, Lugo, Spain (43°14′30.16″N, 7° 32′55.539″W) in September 2013 and 2014. Harvested mature brown fruits were kept in dry paper bags under room laboratory conditions until used. Individual mericarps (seeds) were obtained by mechanical friction and stored dry in plastic Petri dishes at 4°C for 12 weeks in darkness.

Germination Conditions

Eryngium viviparum Gay is an endangered plant and its use is legally limited, being extremely difficult to obtain both fruits and/or seeds, therefore two batches of just 750 seeds (2013) and 1,440 seeds (2014) were used in this study. The initial experiments were adjusted to maximize the number of seeds per treatment (55–100), whereas in the following experiments the treatments were performed with 80 seeds each (see **Table 1**).

Several treatments have been carried out to improve *Eryngium* germination including seed surface sterilization and stratification (cold and warm), to stimulate seed dormancy breaking and to induce embryo growth and development.

Seed Sterilization

Seeds were surface sterilized before (wet stratification) or after (dry stratification) of the dormancy-breaking treatments.

In both cases, seeds were soaked in 2% sodium hypochlorite for 5 min. After, in laminar flow cabinet, seeds were washed with sterile distilled water for three times, and stirred in 50% sulfuric acid for 40 min. Seeds were removed from sulfuric solution, washed again for three times during 5 min, and soaked overnight in sterile distilled water, previous dormancy breaking and incubation experiments.

TABLE 1 | Design of *Eryngium* seed dormancy-breaking and incubation procedures.

		Dormancy-breaking				Incubation				
Treatment	**Seeds (N°)**	**Strat. 4°C (Weeks)**	**Strat. 25°C (Weeks)**	**Moisture condition**	**GA_3 (mg L^{-1})**	**Substrate**	**Temp (°C)**	**GA_3 (mg L^{-1})**	**KIN (mg L^{-1})**	**Time (weeks)**
1	95	8	4	Dry	0	MS	24	0	0	20
2	95	8	4	Dry	0	MS	24	1	0	20
3	95	8	4	Dry	0	MS	24	0	1	20
4	55	8	4	Dry	0	MS	18-13	0	0	20
5	55	8	4	Dry	0	MS	18-13	1	0	20
6	55	8	4	Dry	0	MS	18-13	0	1	20
7	100	8	24	Dry	0	MS	18-13	0	0	20
8	100	8	24	Dry	0	MS	18-13	0	1	20
9	100	8	24	Dry	0	MS	18-13	0	5	20
10	80	0	4	Dry	0	Water	18-13	0	0	10
11	80	0	4	Dry	0	MS	18-13	0	0	10
12	80	0	4	Dry	0	MS	18-13	0	1	10
13	80	0	4	Dry	0	MS	18-13	0	5	10
14	80	0	4	Wet	0	Water	18-13	0	0	10
15	80	0	4	Wet	0	MS	18-13	0	0	10
16	80	0	4	Wet	0	MS	18-13	0	1	10
17	80	0	4	Wet	0	MS	18-13	0	5	10
18	80	0	4	Wet	2	Water	18-13	0	0	10
19	80	0	4	Wet	2	MS	18-13	0	0	10
20	80	0	4	Wet	2	MS	18-13	0	1	10
21	80	0	4	Wet	2	MS	18-13	0	5	10
22	80	8	0	Wet	0	Water	18-13	0	0	10
23	80	8	0	Wet	0	MS	18-13	0	0	10
24	80	8	0	Wet	0	MS	18-13	0	1	10
25	80	8	0	Wet	2	Water	18-13	0	0	10
26	80	8	0	Wet	2	MS	18-13	0	0	10
27	80	8	0	Wet	2	MS	18-13	0	1	10

Seed were previously stored in darkness at 4°C during 12 weeks (MS, Murashige and Skoog medium; GA_3, gibberellic acid, and KIN, kinetin).

Dormancy-Breaking Procedure

On the first batch of seeds, long periods of cold stratification (12 + 8 weeks at 4°C) followed by a variable period of warm stratification (4–24 weeks at 25°C) were tested. All seeds (750) were stratified in dry paper filter in Petri dishes (treatments 1–9; **Table 1**).

Further, with the second batch of seeds, also shorter periods of cold stratification (12 + 0) and warm stratification (0–4 weeks at 25°C) than in 2013 were tested (treatments 10–13). Wet treatments (water or 2 mg L^{-1} GA_3 solutions) were also included (treatments 14–27). In these treatments, seeds were surface sterilized previously.

Incubation Procedure

Once completed dormancy breaking procedures, surface sterilized seeds were sowed in sterilized glass culture vessels on 25 mL MS medium (Murashige and Skoog, 1962) supplemented with GA_3 (0 and 1 mg L^{-1}) or KIN (0, 1, and 5 mg L^{-1}) or in plastic Petri dishes on double filter paper layer moistened with 20 mL sterile distilled water (**Table 1**). Sterilization was carried in autoclave at 121°C for 20 min at 105 kPa.

Incubation was carried out in growth chambers at 24 or 18–13°C thermoperiod (similar to the habitat temperature) and 12/12 h photoperiod (Flux density of 55 μmol m^{-2} s^{-1}) during 10 and 20 weeks. All combinations are shown in **Table 1**.

Germination Test

Germinated and no-germinated seed percentage was calculated after 20 or 10 weeks (**Table 1**) treatments 1–9 and 10–27, respectively. Seeds with longer than 1 mm visible radicle were considered as germinated. No-germinated seeds were dissected by its longitudinal axis and evaluated using a stereomicroscope (Nikon SMZ-U). In order to measure and calculate the embryo and seed length ratio (E:S ratio; Vandelook et al., 2007a), the camera software package (0.7X DXM Lens Nikon) was used.

Another batch of seeds (50 in 2013 and 2014) were stored during 12 weeks in darkness at 4°C, dissected and their ratio E:S calculated in order to know the embryo development in the moment of start the germination treatments. This E:S ratio was used as control.

Once germinated or dissected, seeds were split out into two groups: viable and non-viable seeds. All germinated seeds and

those no germinated but with embryo visible were included as "viable seed" and employed to calculate the following parameters:

- ***Seed germination percentage*: %G** = (N° germinated seeds/N° seeds) * 100
- ***Real seed germination percentage*: %RG** = (N° germinated seeds/N° viable seeds) * 100
- ***No germinated seeds percentage*: %NG** = (N° no germinated viable seeds/N° seeds) * 100
- ***E:S ratio*** = Embryo length/Seed length. Germinated seeds were considered as E:S = 1 and included in the calculation average E:S ratio.

Non-germinated seeds were considered as "non-viable," including embryoless seeds and those completely empty.

Two new parameters were calculated for the non-viable seeds:

- ***Embryoless seeds percentage*: %EL** = (total N° embryoless seeds/total N° seeds) * 100
- ***Empty seeds percentage*: %EM** = (total N° empty seeds/total N° seeds) * 100

Statistical Analysis

Student *t*-test was performed to test the significant difference ($P < 0.05$) of E:S ratio respect to control.

Neurofuzzy Logic

A database with results from 27 treatments was modeled using the commercial neurofuzzy logic software, FormRules v4.03 (Intelligensys Ltd., UK). Neurofuzzy logic combines the learning capabilities of neural networks with the linguistic capabilities of fuzzy logic. Neurofuzzy model allows to model germination results as a function of the several factors studied and predict results for a not study combination of factors. Additionally, it allows expressing the model through simple IF…THEN rules providing understanding and knowledge. Every IF-THEN rule is associated to a membership degree which represents the degree of truth from 0 to 1 (Gallego et al., 2011; Gago et al., 2014; Nezami-Alanagh et al., 2014, 2017).

For the modeling, the inputs were the nine germination variables studied during dormancy breaking (time of stratification at 4°C, time of stratification at 25°C, moisture condition, and GA_3 concentration) and incubation (substrate, temperature, GA_3 concentration, KIN concentration and time) and the outputs were the six germination parameters defined above (%G, %NG, %EL, %EM, %RG, and E:S ratio).

A separate model was developed for each output. The parameters used for modeling by FormRules® are shown in **Table 2**.

TABLE 2 | The training parameters setting with FormRules v3.31.

MINIMIZATION PARAMETERS
Ridge regression factor: 1 e^{-6}
MODEL SELECTION CRITERIA
Structural Risk Minimization (SRM)
C1 = 0.8–0.854 C2 = 4.8
Number of set densities: 2
Set densities: 2. 3
Adapt nodes: TRUE
Max. inputs per SubModel: 4
Max. nodes per input: 15

RESULTS

Seed Sterilization

Field sampled *E. viviparum* seeds presented severe contamination, which was not eliminated by the procedures described for other Apiaceae. Despite of several disinfection procedures as ethanol 70% and sodium hypochlorite 1–2%, *Eryngium* seeds remained highly contaminated (60–100%). Additional strong treatments including seed soak in sodium hypochlorite 2% for 5 min, long rise in distilled water and stir in 50% sulfuric acid for 40 min were necessary to completely eliminate contamination.

Seed Germination

Table 3 presents *E. viviparum* germination percentages (%G) for all the conditions studied. As it can be seen, the germination values are really poor for all the treatments; being the maximum seed germination percentage 27.3% for treatment 2. The highest germination values were reached on treatments 2, 3, and 8 (>20%G), which shared the next conditions: long cold dry stratification period (12 + 8 weeks) without GA_3 and long incubation of 20 weeks (**Table 1**). Treatments 18, 19, 20, 21, and 25, give no germination at all (**Table 3**). Seeds on these treatments were stratified in wet conditions (with 2 mg L^{-1} GA_3) and thermoperiod for 10 weeks (**Table 1**).

Looking for the reasons for those low germination rates, no germinated seeds were dissected. As it can be seen in **Figure 1**, the images show that seeds could be classified as empty (A), embryoless (B), seeds with underdeveloped embryo (C), and with fully developed embryo (D).

Percentages of no germinated seeds (%NG), embryoless (%EL), and empty seeds (%EM) help to explain previous germination percentages (**Table 3**). The overall low germination rate was due to a high number of NG seeds (mean 30.4%) and non-viable seeds (mean 62.6%), from which 25.5% where embryoless and 37.0% empty seeds, respectively (**Table 3**). These results suggest that, only a maximum of 37.4% could be germinated in optimal conditions (7.1 %G + 30.4 %NG).

If the germination percentage is recalculated considering just the viable seeds, named as real seed germination (%RG; Materials and Methods) the results are promising, as some of the treatments reach 89–100% (treatments 2 and 4; **Table 3**).

Table 3 also shows the E:S ratio obtained at the end of each treatment. All treatments have values higher than 0.31 (control E:S ratio from seeds stored during 12 weeks at 4°C) although not significant differences were found for some of the treatments against control (**Table 3**). This results point out the important role of some of the conditions used during dormancy–breaking and incubation procedures on embryo development and justifies the modeling in order to understand which and how they affect germination.

TABLE 3 | Percentage of germination (%G), no germination (%NG), embryoless seeds (%EL), empty seeds (%EM), viable (%G+%NG), non-viable (%EL+%EM) seeds, real germination (%RG), and mean and standard error of the E:S ratio.

Treatment	%G	%NG	%EL	%EM	%Viable	%Non-viable	%RG	E:S ratio (± SE)
1	7.1	14.3	28.6	50.0	21.4	78.6	33.3	0.51 (± 0.25)
2	**27.3**	**0.0**	45.5	27.3	27.3	72.7	**100.0**	**1.00 (± 0.00)***
3	**22.2**	33.3	**0.0**	44.4	55.6	44.4	40.0	0.50 (± 0.21)
4	17.8	2.2	11.1	68.9	20.0	80.0	88.9	0.98 (± 0.02)*
5	12.8	10.3	23.1	53.8	23.1	76.9	55.6	0.71 (± 0.13)*
6	16.0	12.0	8.0	64.0	28.0	72.0	57.1	0.74 (± 0.14)*
7	13.6	27.3	8.0	51.1	40.9	59.1	33.3	0.54 (± 0.06)*
8	**22.2**	20.8	15.3	41.7	43.1	56.9	51.6	0.68 (± 0.06)*
9	15.1	19.2	19.2	46.6	34.2	65.8	44.0	0.62 (± 0.07)*
10	5.4	39.5	34.0	21.1	44.9	55.1	12.1	0.48 (± 0.05)*
11	3.3	36.7	34.0	26.0	40.0	60.0	8.3	0.47 (± 0.05)*
12	1.3	34.2	**36.2**	28.2	35.6	64.4	3.8	0.43 (± 0.04)*
13	4.0	28.7	20.7	46.7	32.7	67.3	12.2	0.57 (± 0.08)*
14	2.1	37.6	27.0	33.3	39.7	60.3	5.4	0.43 (± 0.04)*
15	5.0	43.3	22.7	29.1	48.2	51.8	10.3	0.45 (± 0.04)*
16	4.8	32.9	29.5	32.9	37.7	62.3	12.7	0.49 (± 0.06)*
17	2.8	40.0	27.6	29.7	42.8	57.2	6.5	0.42 (± 0.04)*
18	0.0	35.7	32.2	32.2	35.7	64.3	0.0	0.36 (± 0.02)
19	0.0	38.5	29.4	32.2	38.5	61.5	0.0	0.33 (± 0.01)
20	0.0	42.8	26.9	30.3	42.8	57.2	0.0	0.35 (± 0.01)
21	0.0	42.4	32.6	25.0	42.4	57.6	0.0	0.36 (± 0.01)*
22	2.3	32.6	28.0	37.1	34.9	65.1	6.6	0.45 (± 0.06)*
23	1.7	28.2	31.6	38.5	29.9	70.1	5.8	0.49 (± 0.08)*
24	1.1	39.5	29.4	29.9	40.7	59.3	2.8	0.37 (± 0.03)
25	0.0	41.7	30.3	28.0	41.7	58.3	0.0	0.37 (± 0.01)*
26	1.7	41.0	28.3	28.9	42.8	57.2	4.1	0.40 (± 0.04)*
27	1.1	45.4	30.5	23.0	46.6	53.4	2.5	0.37 (± 0.03)
Mean	**7.1**	**30.4**	**25.5**	**37.0**	**37.4**	**62.6**	**22.1**	**0.51 (± 0.03)**

Treatment with E:S different significantly with control (0.31) at $p < 0.05$ is indicated (*).

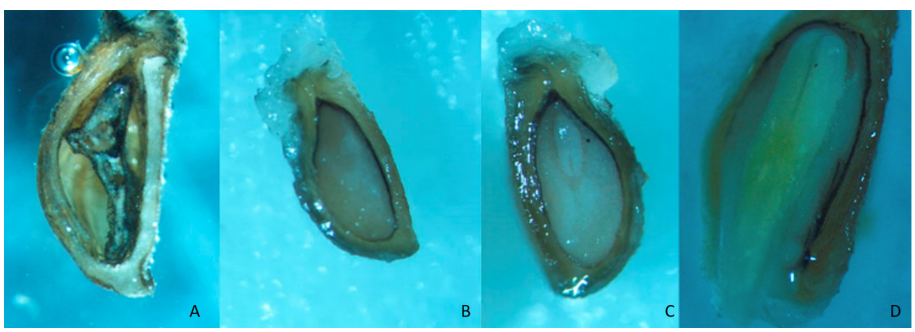

FIGURE 1 | Dissected *Eryngium* seeds empty **(A)**, embryoless **(B)**, with underdeveloped embryo **(C)** and fully developed embryo **(D).**

Neurofuzzy logic succeeded in simultaneously modeling the six germination parameters. **Table 4** shows the inputs that explain the variability of each output together with the quality parameters for the models obtained: correlation coefficients (R^2) and ANOVA parameters (calculated f ratio, degrees of freedom and f critical for $\alpha = 0.01$). As it can be seen R^2-values ranged from 74.85 to 95.92% indicating high model predictabilities. Moreover, ANOVA f-ratio values were always higher than the corresponding f critical values ($\alpha < 0.01$) indicating their accuracy. It is interesting to note several general features from these results: (a) Only 6 out of 9 inputs help to explain the variability of the outputs studied, (b) The factor time of

TABLE 4 | Critical factors from the neurofuzzy logic Train R^2 and ANOVA parameters for training [*f* ratio, degree of freedom (df1: model and df2: total) and *f* critical value for $\alpha < 0.01$] for each output.

Outputs	Critical factors	Train set R^2	*f* ratio	df1, df2	*f* critical
%G	**Time**	79.69	47.0883	2, 26	5.53
%NG	**Time** GA_3 (I)	74.85	22.8146	3, 26	4.64
%EL	**Time** Temp × KIN GA_3 (I)	85.97	20.4282	6, 26	3.59
%EM	**Time** Temp Strat. 25°C GA_3 (I)	81.32	18.278	5, 26	5.80
%RG	**Strat. 25°C × Time** Temp × GA_3 (I) GA_3 (D-B)	95.92	52.8306	8, 26	3.29
E:S	**Temp × GA_3 (I)** Time Strat. 25°C GA_3 (D-B)	90.39	25.5405	7, 26	3.42

Concentration of GA_3 during dormancy breaking (D-B) or incubation (I) period. The inputs with stronger effect on each output have been highlighted.

incubation has been highlighted by the model as important for all the parameters, (c) %RG and E:S are explained by the same set of inputs including two from dormancy breaking phase (Strat. 25°C and GA_3) and three from incubation (Time, GA_3 and Temp), and (d) The variation of the parameters %NG, %EL, and %EM, which are associated with low percentages of germination, are explained by the time of incubation and the concentration of GA_3 during the incubation period, but also involves other variables such as the time of stratification at 25°C or the temperature and the concentration of KIN during incubation.

IF-THEN rules generated by the neurofuzzy logic software with an associated membership degree are shown in **Table 5**. All factors included in the models were fuzzyficated as low or high indicating a linear effect on germination parameters in the range of the study, independently if they have a significant effect alone or in interaction with other factor. Those rules greatly simplify the process of understanding the cause-effect relationship among factors and germination parameters. As an example, the percentage of germination variability is explained by just one factor: time of incubation (**Table 4**). IF time of incubation is low, THEN the %G will be low (Rule 1; **Table 5**). The membership 0.93 indicates that in this condition the percentage of germinated *Eryngium* seeds predicted by the model will be close to the lowest % G values in the database. Therefore, 20 weeks of incubation promotes higher germination percentages than 10 weeks, as it is for treatments 2, 3, and 8 with the highest %G (highlighted in **Table 3**).

On the contrary, the %NG variability is explained by two factors: time and concentration of GA_3 during the incubation period (**Table 4**). Neurofuzzy logic highlights the key role of incubation time but logically, in the opposite way than for %G (**Table 5**): if low incubation time is used, the percentage of no germination for *Eryngium* seeds is high (rule 3; membership 0.90) and if the time of incubation is high then %NG is low (rule 4; 0.95). In the same sense if the plant growth regulator GA_3 is present in the incubation solution (1 mgL^{-1}) then the %NG will be low. These rules explain for example, the high value for %G together with low value for %NG obtained for treatment 2 (**Table 3**).

Two kinds of non-viable seeds were detected: embryoless but with endosperm (EL; **Figure 1B**) or without endosperm (EM; **Figure 1A**). The percentage of both in *Eryngium* seeds is determined by incubation time, but in a different way (**Table 5**). While a long period of incubation promotes always low embryoless seeds percentages (rule 8; membership 1.0), also promotes high percentages of empty seeds (rule 16; 1.0). The interaction of high temperature and high kinetin concentration gives low embryoless seeds percentages (rule 14; 1.0), as can be seen when compared treatments 3 (24°C) vs. 12 (18–13°C) in **Table 3**. The presence (high) of GA_3 during incubation (rules 10 and 22) also reduces the %EL and %EM, in agreement with the results obtained for %NG (rule 6).

The percentage of real seed germination (RG) variability can be explained by a selection of five inputs (the interaction of time of stratification at 25°C and time of incubation, the interaction of temperature and concentration of GA_3 during incubation and the concentration of GA_3 during dormancy breaking process). From the rules corresponding to RG parameter (**Table 5**) it is easy to deduce that; (a) a short period of warm stratification (low) combined to a long period of incubation (high weeks) rendered, always, high %RG (rule 25; membership 1.0); (b) high temperature in combination with 1 mg L^{-1} GA_3 during incubation also promotes high % real germination (rule 30; 1.0); and (c) the addition of GA_3 during dormancy breaking period, fully inhibits *Eryngium* seed germination (rules 31–32). All this conditions can be observed in treatment 2 (**Table 1**) with high %RG (100%; **Table 3**).

High E:S ratio favor seed germination. Neurofuzzy pointed out, that the interaction high incubation temperature and 1 mg L^{-1} GA_3 promotes embryo growth (rule 38; membership 1.0) as in treatment 2 (**Table 3**). Long periods of incubation (rule 34; 1.0) together with short warm stratification periods (rule 39; 0.96) improve E:S (**Table 5**). On the contrary, the presence of GA_3 in the wet solution during the break dormancy procedure reduce E:S ratio (rule 42; 1.0). These results are in fully agreement with those described for %RG.

DISCUSSION

According to literature review, no disinfection or general antiseptic procedures such as ethanol 70% for 30 s followed by sodium hypochlorite at low concentration 1% for 5–10 min were enough for surface seed sterilization in Apiaceae family (Walmsley and Davy, 1997; Vandelook et al., 2008; Thiem et al., 2013). Those typical procedures did not work at all for *E. viviparum* seeds. All seeds revealed severe contamination by

TABLE 5 | Rules generated by neurofuzzy logic.

Rules		Strat. 25°C	GA_3 (D-B)	Temp	GA_3 (I)	KIN	Time		G	NG	EL	EM	RG	E:S	Membership degree
1	**IF**						**Low**	**THEN**	**Low**						**0.93**
2							**High**		**High**						**0.63**
3	**IF**						**Low**	**THEN**		**High**					0.90
4							**High**			**Low**					0.95
5					Low					High					0.77
6					High					Low					0.82
7	**IF**						**Low**	**THEN**			**High**				**0.54**
8							**High**				**Low**				**1.00**
9					Low						Low				1.00
10					High						Low				0.61
11				Low		Low					High				1.00
12				Low		High					High				1.00
13				**High**		**Low**					**High**				**1.00**
14				**High**		**High**					**Low**				**1.00**
15	**IF**						**Low**	**THEN**				**Low**			**1.00**
16							**High**					**High**			**1.00**
17				Low								High			0.89
18				High								Low			1.00
19		Low										High			0.98
20		High										Low			1.00
21					Low							High			0.67
22					High							Low			1.00
23	**IF**	**Low**					**Low**	**THEN**					**Low**		**1.00**
24		High					Low						Low		1.00
25		**Low**					**High**						**High**		**1.00**
26		High					High						Low		0.59
27				Low	Low								Low		0.61
28				High	Low								Low		1.00
29				Low	High								Low		1.00
30				HIgh	High								High		1.00
31			Low										Low		0.51
32			High										Low		0.71
33	**IF**						Low	**THEN**						Low	1.00
34							High							High	1.00
35				Low	Low									High	0.60
36				**High**	**Low**									**Low**	**1.00**
37				Low	High									Low	1.00
38				**High**	**High**									**High**	**1.00**
39		Low												High	0.96
40		High												Low	1.00
41			Low											Low	0.54
42			High											Low	1.00

The inputs with the highest membership degree (stronger effect) on each output indicates by the model are highlighted.

fungi during germination and none were able to germinate. Only after application of strong antiseptic procedures including sulfuric acid, a method commonly used to surface-sterilize seeds heavily contaminated with fungi (Latchs and Christensen, 1985; Siegel et al., 1987), we succeeded in achieving the total elimination of fungal contamination.

Poor germination rate is a common problem in the Apiaceae family (Robinson, 1954; Ojala, 1985; Baskin and Baskin, 2014).

Eryngium genus is not an exception. *Eryngium maritimum* (Walmsley and Davy, 1997; Necajeva and Ievinsh, 2013), *Eryngium planum* (Thiem et al., 2013) and *Eryngium foetidum* (Mozumder et al., 2011; Mozumder and Hossain, 2013), have shown lower than 20, 12, and 10% germination respectively, without dormancy breaking treatments. Little information on *Eryngium viviparum* is available. Our results ranging from 0 to 27% of germination are in agreement with Magnanon and coworkers that reported low germination percentages (10–40%) on populations from France and Spain (Magnanon et al., 2012).

Low germination rates in the Apiaceae family have been correlated with the next three causes (Robinson, 1954; Ojala, 1985; Baskin and Baskin, 2014): (1) presence of high percentage of non-viable seeds without embryo; (2) presence of high percentage of seed with underdeveloped embryos; and (3) presence of dormant seeds.

In agreement with the first cause our results showed a high non-viable seeds percentage (62.5%) close to other Apiaceae as *Anethum graveolens* (ranged 39–62%; Robinson, 1954) or *Anthriscus caucalis* (49%; Rawnsley et al., 2002). Among the potential causes for non-viable seeds (zygote degeneration, death of embryo, mutations, etc.; Baskin and Baskin, 2014), two have been described for Apiaceae family: (1) insect infestation; these seeds have an endosperm but not embryo (Flemion and Henrickson, 1949) and (2) self-pollinated umbels; seeds produced in these umbels are usually empty (without endosperm and embryo; Ojala, 1985). In this work, the high percentage of non-viable seeds have been described as embryoless (25.5% EL) and empty (37% EM), supporting the hypothesis that the poor germination percentage of *E. viviparum* seeds can be explained as a consequence of the high percentage of non-viable seeds due to insect infestation and self-pollinated umbels.

The second cause of low germination in Apiaceae is the presence of underdeveloped embryos at the moment of dispersal (Martin, 1946). The embryo needs to grow up to a critical length before germination. E:S ratio was investigated to check the presence of underdeveloped embryos in *E. viviparum* (**Table 3**). The E:S ratio seeds stored during 12 weeks at 4°C in darkness, used as control, averaged 0.31, similar to those values found for other Apiaceae species as *Torilis japonica* with a ratio 0.25, *Angelica sylvestris* with 0.29, or *Selinum carvifolia* with 0.31 at harvest. However, after 20 weeks at 5°C, those embryos growth until 0.29, 0.39, and 0.34, respectively, a little higher than the E:S ratio at harvest (Vandelook et al., 2007a, 2008). This delay in seed germination due to the underdeveloped and differentiated embryo is called morphology dormancy (MD, Nikolaeva, 1977), and those Apiaceae has been characterized as MD seed (Vandelook et al., 2007a,b, 2008). Our results, clearly demonstrated a high percentage 30.4% of underdeveloped embryo seeds suggesting that *E. viviparum* also showed MD dormancy, in agreement with other *Eryngium* sp. (Necajeva and Ievinsh, 2013). Seeds with MD, as Apiaceae, need specific conditions as a moist substrate, suitable temperature and photoperiod as growth embryo requirements (Baskin and Baskin, 2014). If seeds remain under these conditions, embryos should growth and germinate in 4 weeks or less. No precise protocol to overcome this problem (the need of right conditions enabling embryo growth and development until reach a critical size to germinate) has been previously described in the literature for *E. viviparum*.

The third cause for low germination rates in Apiaceae is the existence of an additional physiological mechanism of dormancy, which also inhibits germination (Baskin and Baskin, 2004). Some Apiaceae present both dormancies morphological (MD) and physiological (PD) at the same time, known as morphophysiological dormancy (MPD), and need a considerably longer period for germinate than MD seeds (Baskin and Baskin, 2004). The combination of cold and warm stratification can be effective for break MPD dormancy and allow the embryo to growth. Warm stratification was effective in some species of *Osmorhiza* and *Erythronium* genus (Baskin et al., 1995). In addition, short warm stratification increases the seed sensitivity to GA_3 improving germination rate in *E. maritimum* and suggesting a key role of GA_3 to overcome the dormancy (Vandelook et al., 2008; Necajeva and Ievinsh, 2013). *E. viviparum* presents low percentage of seeds (mean 7.1%) that germinate after breaking the MPD. Interesting, some treatment, such as 2, reached 27.3%G and 100%RG, which means that all viable seeds were able to break MPD dormancy and germinate.

To understand the physiological mechanism of dormancy breaking, a representative study of seed germination require test several factors, which implies many different treatments and replicates, and finally large batches of seeds. Those requirements are essential for an accurate interpretation of the cause-effect of those factors and their interactions on seed germination (International Seed Testing Association, 1985). As stated above, *E. viviparum* is an endangered species and therefore, no large seed batches are available. Based on these limitations, two experiments including just 27 treatments were proposed. We test the most important factors well known as dormancy breaking in Apiaceae such as cold and warm stratification and two PGR (GA_3 and KIN) well known as inducers of embryo development (Vandelook et al., 2008; Mozumder and Hossain, 2013; Necajeva and Ievinsh, 2013). The authors are aware that the design space was not well-sampled, which hinders a conventional statistical treatment to analyse the results and draw conclusions. Neural networks has demonstrated to be a practical approach to deciphering the key factors in several biological process and an excellent alternative to conventional statistical methods (Gago et al., 2010a,b; Gallego et al., 2011; Nezami-Alanagh et al., 2014; Arab et al., 2016). Advantageously, neurofuzzy logic technology allows working with not well-defined design spaces and different kind data at the same time (Nezami-Alanagh et al., 2017). In this paper, that technology was used to reduce the number of treatments and therefore, the needed seeds, without losing key information to extract scientific conclusions.

Neurofuzzy logic succeeded in modeling all outputs simultaneously with high predictability and accuracy (**Table 3**). Additionally, it allowed obtaining a set of rules that explain the cause-effect among the factors (inputs) and the germination parameters obtained (**Table 4**). The model pinpointed that the

factor time of incubation was the most significant factor *for Eryngium* seed germination; long periods (around 20 weeks) are strongly recommended to obtain high %G, low %NG, low %EL, high E:S, and high %RG, but in this case in combination with low warm stratification. In fact only after 20 weeks of incubation the highest real germination rates can be reached (see treatment 2 and 4; **Table 3**). Interesting, also with high incubation periods a high percentage of empty seeds was obtained, suggesting a high correlation between endosperm degradation and duration of the germination period. The model also identified the important role of the interaction between incubation temperature and GA_3 concentration. If temperature is around 24°C and 1 mg L^{-1} GA_3 is present, the highest real germination and E:S was obtained. The third most important factor was the factor stratification at 25°C. Low period of warm stratification (4 weeks) is preferred to obtain the highest % of real germination and E:S ratio (1.00 membership). Some authors (Necajeva and Ievinsh, 2013) demonstrated that seed of *E. maritimum* seed germinated to a high final germination percentage only after 12–16 weeks cold stratification. Our model suggests that *E. viviparum* presents seeds with MDP, and needs to break dormancy. Long incubation time (20 weeks) combined with low warm stratification (4 weeks) at continuous high temperature 24°C and 1 mg L^{-1} GA_3 are strongly recommended.

AUTHOR CONTRIBUTIONS

MA: Performed the experiments; PR-R: Contributed with reagents/materials; ML and PG: Contributed modeling/analysis tools; PG and MB: Conceived and designed the experiments. All authors contributed to writing of the manuscript.

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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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ORIGINAL ARTICLE

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

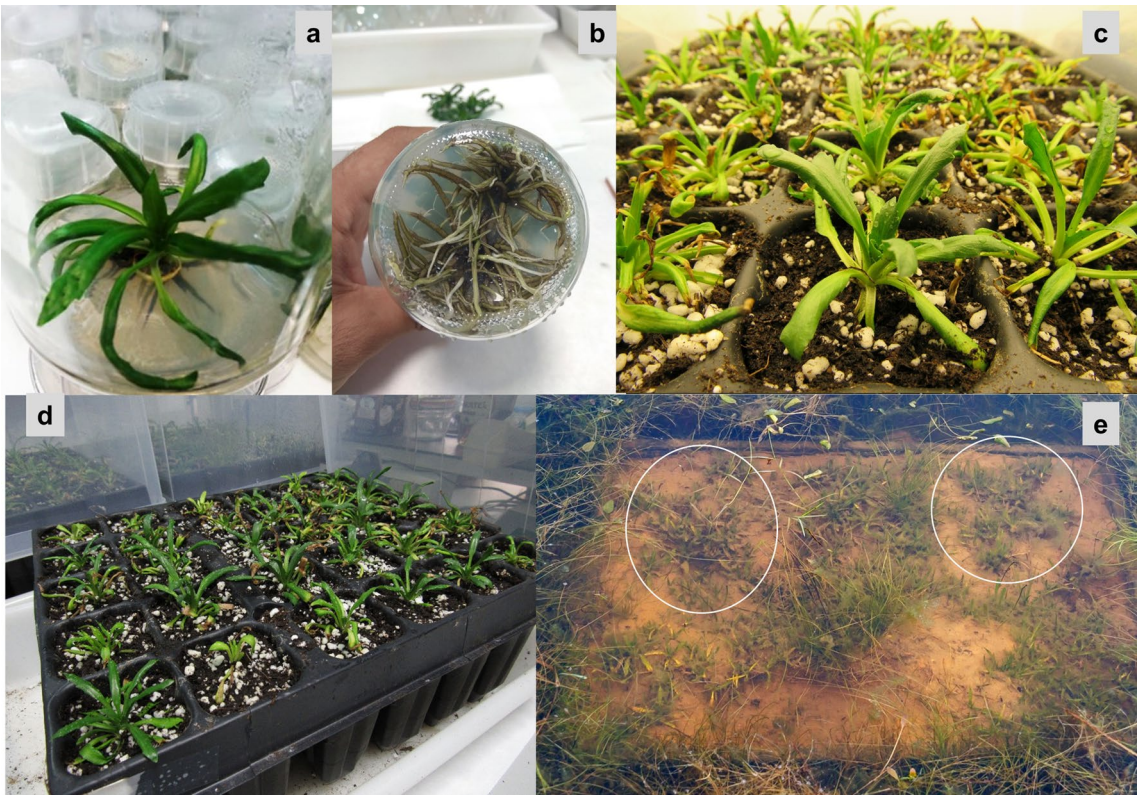

Fig. 1 In vitro-cultured *E. viviparum* showing the typical rosette development on the multiplication stage (**a**). New roots formed after 30 days on rooting stage (**b**). *E. viviparum* plantlets placed in pots with peat and perlite (1:1 v/v) substrate and covered with plastic boxes during acclimatization 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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Phenolic composition and biological activities of the *in vitro* cultured endangered *Eryngium viviparum* J. Gay

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ABSTRACT

Eryngium viviparum is an endangered species that requires management efforts based on complementary *ex situ* conservation strategies, such as *in vitro* culture. This study was carried out to evaluate the phenolic composition and the antioxidant, cytotoxic, and antimicrobial properties of *E. viviparum* aerial and root parts obtained by this micropropagation technique. The HPLC-DAD-ESI/MS^n analysis showed that phenolic compounds were more abundant in the root (102 ± 4 mg g^{-1} extract) than in the aerial part (40.6 ± 0.8 mg g^{-1} extract) of the plant. The major compound was *trans* rosmarinic acid, followed by *trans* 3-*O*-caffeoylquinic acid. The root extract also showed higher antioxidant activity, with a result close to that of trolox in the thiobarbituric acid reactive substances (TBARS) formation inhibition assay, and a moderate toxicity to lung (NCI-H460), breast (MCF-7) and liver (HepG2) tumour cells. It was also more effective than ketoconazole against *Penicillium ochrochloron*. In turn, the aerial part extract inhibited *Salmonella typhimurium* more effectively than ampicillin. This study highlights *E. viviparum* as an unexplored source of bioactive compounds with potential application in the food, pharmaceutical, and other industrial sectors. Consequently, it promotes the interest of conserving this endangered species.

1. Introduction

Eryngium viviparum J. Gay (Fam. Apiaceae) is an endemic plant from the northwest of the Iberian Peninsula and France. Its current population is dramatically small and faces a high risk of extinction, being included in the International Union for Conservation of Nature's red list of threatened species since 2011 (Aguiar, 2003; Lansdown, 2011). To promote their protection, usual *in situ* conservation strategies have been implemented, such as management of wild populations and protection of natural habitats (Bañares et al., 2004). Currently, in Spain and Brittany (France), only policies based on *in situ* conservation are commonly used (Rascle et al., 2018; Romero et al., 2004). However, in some critical situations, it is necessary to resort to *ex situ* strategies such as *in vitro* plant tissue culture.

Micropropagation is an *in vitro* culture technique that allows the large-scale multiplication of plant species from excised plant parts within a short span of time, as well as for the storage and preservation of germplasm. The obtained *in vitro*-cultured plants (clones of the original mother plant) can be used in plant reintroduction programs and for research purposes, without damaging the existing populations (Ayuso et al., 2019; Engelmann, 2011; Sarasan et al., 2006). Plant tissue culture is also used to stimulate the synthesis and accumulation of secondary metabolites in the plant tissues, including alkaloids, terpenes, and phenolic compounds (Atanasov et al., 2015; Dias et al., 2016).

Several *Eryngium* species have been used in traditional medicine for the treatment of various human physiological disorders due to their bioactive chemical constituents. Aerial and root parts are used in the treatment of skin disorders and kidney stones, and to regulate arterial pressure (Wang et al., 2012). Infusions of these parts are also used to treat tapeworms and pinworms, digestive problems, and headache, and as a diuretic (Erdem et al., 2015; Vukic et al., 2018). Current research with plants of this genus has confirmed the presence of phytochemicals with pharmacological activities, specifically flavonoids and phenolic acids (Erdem et al., 2015; Wang et al., 2012), which have been reported as one of the main groups of phytochemicals with antioxidant,

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antimicrobial, and cytotoxic properties (Belkaid et al., 2006; Gugliucci and Bastos, 2009; Petersen and Simmonds, 2003).

Nowadays, the interest in natural and bio-based ingredients has increased due to the high market demand from the consumer and the food industry, among other sectors. However, the discovery of new bioactive compounds and the use of more sustainable sources at an industrial scale face some challenges related to the rational exploitation of natural resources and biodiversity (Atanasov et al., 2015; Lavergne et al., 2005; Li and Vederas, 2009). Although *E. viviparum* is a threatened species, the conditions for its germination and *in vitro* culture have already been established in recent studies (Ayuso et al., 2019, 2017); nevertheless, to the best of authors' knowledge, the phenolic profile and bioactive properties of this plant remain unknown. Therefore, this work was carried out to determine the composition in phenolic compounds and the antioxidant, antimicrobial, and cytotoxic activities of *E. viviparum* aerial and root parts obtained by *in vitro* culture.

2. Material and methods

2.1. Plant material and in vitro culture

Samples of *E. viviparum* were obtained from *in vitro* seed germination as formerly described by Ayuso et al. (2017). Briefly, the seedlings were cultured in MS basal medium, prepared as described by Murashige and Skoog (1962), supplemented with 0.1 mg L^{-1} indole-3-butyric acid, 3% sucrose (*w/v*), and solidified with 1% agar (*w/v*) at pH 5.8. Cultures were incubated at 24 ± 1 °C with photoperiod (16/8; light/darkness). *E. viviparum* grows as a basal rosette and form shoot cluster on *in vitro* culture. Shoots clusters were divided into single shoots every 5 weeks and subculture to a fresh medium (Ayuso et al., 2019). The aerial parts and roots from fifth to eight subculture were collected and then freeze-dried (Telstar Cryodos, Telstar Industrial SL, Terrassa, Spain), reduced to powder, and stored at −20 °C.

2.2. Preparation of hydroethanolic extracts

Samples (~1 g) underwent to solid-liquid extraction twice with 80% ethanol (30 mL) for 1 h at 150 rpm and room temperature, as described by Bessada et al. (2016). After filtration though Whatman paper No. 4, ethanol was separated under pressure in a rotary evaporator (Büchi R-210, Flawil, Switzerland) and the aqueous phase was freeze-dried.

2.3. Analysis of phenolic compounds

The extracts were re-dissolved in 20 % ethanol at 5 mg mL^{-1} and filtered through 0.22 µm syringe filters. The HPLC-DAD-ESI/MS^n equipment and chromatographic conditions were formerly described by Bessada et al. (2016), as well as the identification and quantification procedures. Seven-level calibration curves (25–800 µg mL^{-1} for quercetin-3-*O*-glucoside and 2.5–80 µg mL^{-1} for the other standards were constructed for the standards rosmarinic acid y = –652,903 + 191291x; r^2 = 0.999; LOD = 0.15 µg mL^{-1}; LOQ = 0.68 µg mL^{-1}, chlorogenic acid y = –161,172 + 168823x; r^2 = 0.999; LOD = 0.20 µg mL^{-1}; LOQ = 0.68 µg mL^{-1}, caffeic acid y = 406,369 + 388345x; r^2 = 0.994; LOD = 0.78 µg mL^{-1}; LOQ = 1.97 µg mL^{-1}, ferulic acid y = –185,462 + 633126x; r^2 = 0.999; LOD = 0.20 µg mL^{-1}; LOQ = 1.01 µg mL^{-1}), and quercetin-3-*O*-glucoside y = –160,173 + 34843x, r^2 = 0.999; LOD = 0.21 µg mL^{-1}; LOQ = 0.71 µg mL^{-1}) and used in quantification. The results were expressed as mg/g dry extract.

2.4. Evaluation of biological activities

2.4.1. Antioxidant activity

Two *in vitro* assays were performed to measure the extracts capacity to inhibit the formation of thiobarbituric acid reactive substances (TBARS) and the oxidative haemolysis (OxHLIA) as described by Lockowandt et al. (2019), using porcine brain cells and sheep erythrocytes as oxidizable subtracts, respectively. Trolox was the positive control. The results were given as IC_{50} values (µg mL^{-1}) (Δ*t* of 30 min and 60 min were selected for the OxHLIA).

2.4.2. Cytotoxic activity

The extracts (50–400 µg mL^{-1} in ultrapure water) were tested against NCI-H460 (non-small cell lung carcinoma), MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) human tumour cells by the sulforhodamine B assay (Guimarães et al., 2013a). A non-tumour PLP2 cell line (porcine liver primary cells) was also tested to evaluate possible hepatotoxicity. Ellipticine was the positive control. The results were given as GI_{50} values (µg mL^{-1}).

2.4.3. Antimicrobial activity

The extracts (0.10–20 mg mL^{-1} in water) were tested against *Bacillus cereus* (food isolate), *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (NCTC 7973), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030), and *Salmonella typhimurium* (ATCC 13311) by the microdilution and *p*-iodonitrotetrazolium violet methods (Heleno et al., 2013). *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 36839), and *Penicillium verrucosum* var. *cyclopium* (food isolate) were also tested. The Institute for Biological Research "Sinisa Stanković", Serbia, provided the microorganisms. Positive controls: streptomycin and ampicillin for the antibacterial activity; ketoconazole and bifonazole for the antifungal activity. The results were given as minimum inhibitory, bactericidal, and fungicidal concentrations (MIC, MBC, and MFC, respectively; mg mL^{-1}).

2.5. Statistical analysis

All experiments were performed in triplicate. Statistical tests were performed at a 5% significance level using SPSS Statistics (IBM SPSS Statistics for Windows, 22.0. Armonk, NY: IBM Corp.). Differences among samples were assessed by a Student's *t*-test (for the phenolic compounds content) or a one-way analysis of variance (ANOVA) (for the bioactivities).

3. Results and discussion

3.1. Phenolic composition

The phenolic profiles of the hydroethanolic extracts of *in vitro*-cultured *E. viviparum* aerial and root parts are shown in Fig. 1. Chromatographic data related to the compounds characterization process, tentative identities and contents are given in Table 1. Fourteen phenolic compounds were detected and grouped into phenolic acids (peaks 1–10) and flavonoids (peaks 11–14). Phenolic acids were the predominant compounds in both plant organs, with 38.3 ± 0.8 mg g^{-1} and 102 ± 4 mg g^{-1} of extract of aerial and root parts, respectively; while flavonoids were quantifiable just in the aerial part (2.24 ± 0.09 mg g^{-1} extract).

Peak 9 was identified as *trans* rosmarinic acid based on its mass spectrum (Barros et al., 2013) with a pseudo molecular ion $[M-H]^-$ at *m/z* 359 and fragment ions at *m/z* 197, 179 and 161 (Table 1); its identity was set by comparison with the commercial standard. This phenolic acid was the major compound found in this endangered species, which corresponded to 71.4% and 78.7% of the total phenolic compounds quantified in aerial and root parts, respectively. Lower levels of rosmarinic acid than those found in the *E. viviparum* root have been reported in other Lamiaceae, such as rosemary (*Rosmarinus*

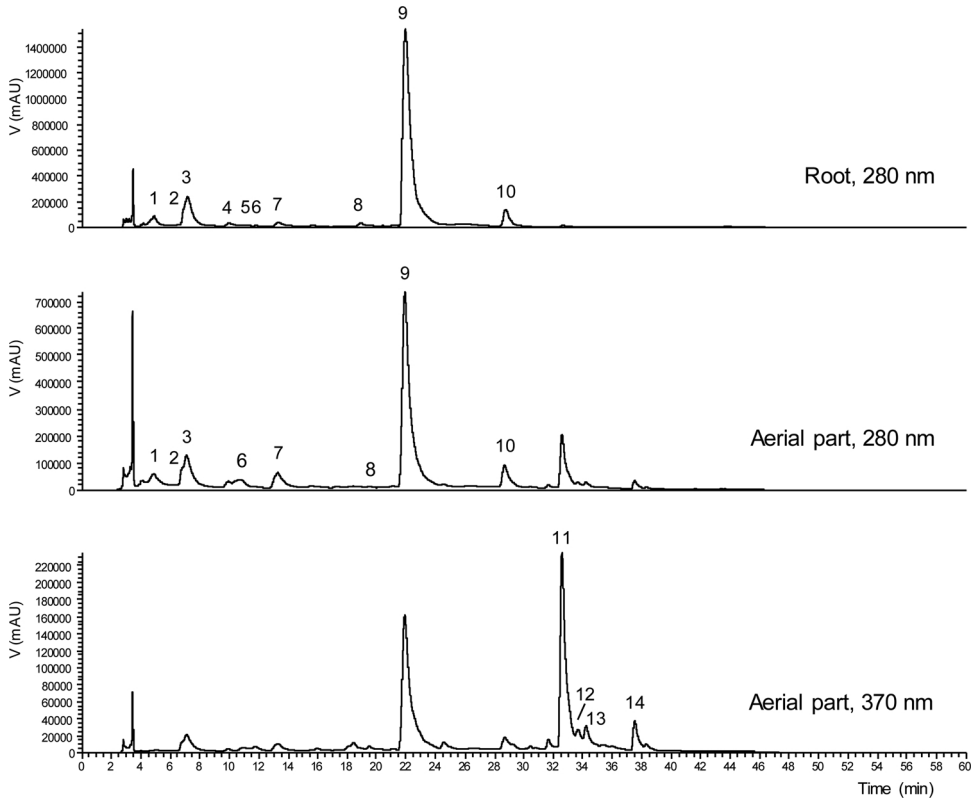

Fig. 1. HPLC phenolic profile of hydroethanolic extracts of *in vitro* cultured *E. viviparum* aerial and root parts. See Table 1 for peak identification.

officinalis L.; 6.9 ± 0.2 mg g^{-1} extract; Gonçalves et al. (2019), and 68.5 mg g^{-1} extract; Ribeiro et al. (2016)) and spearmint (*Mentha spicata* L. 73.4 ± 0.8 mg g^{-1} extract; Chrysargyris et al. (2019)).

Peak 1 ($[M-H]^-$ at *m/z* 197) was identified as 3-(3,4-dihydroxyphenyl)-lactic acid, also known as salvianic acid A or "Danshensu", based on its characteristic fragmentation pattern found in the literature (Zeng et al., 2006). Peak 4 ($[M-H]^-$ at *m/z* 179) was identified as caffeic acid according to its retention time and UV–vis and

Table 1
Phenolic compounds identified and levels quantified in the hydroethanolic extracts of *E. viviparum* root and aerial part. It is presented the retention time (Rt), the wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data ($M-H^-$), and relative abundances of fragment ions (MS^2).

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Content (mg g^{-1} extracts)		Statistics[x]
						Root	Aerial part	*p*-value
1	4.88	281	197	179(100), 135(5)	3-(3,4-Dihydroxyphenyl)-lactic acid[1]	2.41 ± 0.05	1.24 ± 0.05	0.002
2	6.86	326	353	191(100), 179(6), 161(5), 135(5)	*cis* 3-*O*-Caffeoylquinic acid[2]	2.65 ± 0.03	1.25 ± 0.05	0.001
3	7.14	326	353	191(100), 179(6), 161(5), 135(5)	*trans* 3-*O*-Caffeoylquinic acid[2]	10.2 ± 0.4	3.65 ± 0.09	0.002
4	9.99	324	179	135(100)	Caffeic acid[3]	0.24 ± 0.01	tr	–
5	11.19	287/ sh331	357	313(100), 269(39), 203(41), 159(5), 109(5)	Prolithospermic acid (isomer)[1]	1.35 ± 0.05	nd	–
6	11.89	293/ sh332	357	313(100), 269(39), 203(39), 159(5), 109(5)	Prolithospermic acid (isomer)[1]	1.30 ± 0.06	1.48 ± 0.07	0.104
7	13.34	326	367	193(7), 191(100), 173(5), 134(5)	5-*O*-Feruloylquinic acid[4]	0.56 ± 0.02	0.73 ± 0.02	0.014
8	18.9	325	515	353(100), 191(5), 179(69), 161(5), 135(6)	3,5-*O*-Dicaffeoylquinic acid[2]	1.30 ± 0.06	0.28 ± 0.01	0.002
9	21.94	327	359	197(27), 179(36), 161(100), 135(5)	*trans* Rosmarinic acid[1]	80 ± 3	29.0 ± 0.7	0.002
10	28.75	328	373	355(38), 197(13), 193(6), 179(100), 161(62), 135(65)	Feruloyl-hydrocaffeic acid[4]	1.72 ± 0.08	0.73 ± 0.02	0.003
11	32.66	266/ sh347	475	299(100), 175(10)	Tectorigenin-*O*-glucuronide[5]	tr	2.24 ± 0.09	–
12	33.61	266/ sh340	547	503(100), 461(5), 299(5)	Tectorigenin-malonyl-hexoside[5]	nd	tr	–
13	34.21	266/ sh345	547	503(100), 461(5), 299(5)	Tectorigenin-malonyl-hexoside[5]	nd	tr	–
14	37.51	344	517	457(5), 299(100), 285(5), 217(14)	*O*-Methylluteolin-*O*-(acetyl) glucoronide[5]	nd	tr	–
					Σ Phenolic acids	102 ± 4	38 ± 0.8	0.002
					Σ Flavonoids	–	2.24 ± 0.09	–
					Σ Phenolic compounds	102 ± 4	40.6 ± 0.8	0.002

Standards used in the quantification: ([1])- rosmarinic acid, ([2])- chlorogenic acid, ([3])- caffeic acid, ([4])- ferulic acid, and ([5])- quercetin-3-*O*-glucoside. tr: traces; nd: not detected.

[x] A *t*-student test was applied to evaluate statistically significant differences ($p < 0.05$) between samples.

mass characteristics in comparison with the commercial standard.

Peaks 2 and 3 ([M-H]$^-$ at *m/z* 353) were identified as *cis* and *trans* 3-*O*-caffeoylquinic acid, respectively (Zheng et al., 2017), yielding the base peak at *m/z* 191 and ions at *m/z* 179, 161 and 135. The *trans* isomer was the second most abundant quantified phenolic compounds, with aerial and root parts containing 3.65 ± 0.09 mg g^{-1} and 10.2 ± 0.4 mg g^{-1} of extract, respectively.

Both rosmarinic and caffeoylquinic acids were previously reported as the main phenolic acids found in *Eryngium* spp. (Kikowska et al., 2014; Vukic et al., 2018). Rosmarinic acid is a well-known antioxidant with several biological properties, including antibacterial, antiviral, anti-inflammatory, antidiabetic, and anticarcinogenic activities and neuroprotective and cardioprotective effects (Nabavi et al., 2015; Nunes et al., 2015). This compound has major structural features that may contribute to the *in vitro* activities, including a OH group, two phenol − OH groups, a C−C bond, an alkoxy group, and an ester moiety (Taguchi et al., 2017).

Peaks 5 and 6 ([M-H]$^-$ at *m/z* 357) were identified as prolithospermic acid isomers (Grzegorczyk-Karolak et al., 2018), a product of salvianolic acid B degradation often found in the Lamiaceae family (Fecka et al., 2007; Zhou et al., 2011). Peak 7 ([M–H]$^-$ at *m/z* 367) and peak 8 ([M–H]$^-$ at *m/z* 515) were assigned as 5-*O*-feruloylquinic acid and 3,5-*O*-dicaffeoylquinic acid, respectively (Guimarães et al., 2013b).

Regarding flavonoids, a glucuronide-conjugated of tectorigenin, tectorigenin-*O*-glucuronide ([M-H]$^-$ at *m/z* 475), was identified in both organs of *E. viviparum*, being particularly abundant in the aerial part (2.24 ± 0.09 mg g^{-1} extract); only a trace amount was detected in the root extract. Tectorigenin was previously reported in flowers of *Pueraria thomsonii* Benth. and rhizomes of *Belamcanda chinensis* (L.) DC. (Wang et al., 2013). According to Applová et al. (2017), this isoflavone is a more potent antiplatelet compound than acetylsalicylic acid. It has also been shown to exert anti-proliferative effects on tumour cells (Thelen et al., 2005). To the best of the authors' knowledge, this is the first study reporting the presence of tectorigenin in *Eryngium* and Apiaceae spp.

Traces of tectorigenin-malonyl-hexoside (peaks 12 and 13) and *O*-methylluteolin-*O*-(acetyl)glucoronide (peak 14) were detected just in the aerial part of *E. viviparum*.

3.2. In vitro biological activities

3.2.1. Antioxidant activity

The results of the two antioxidant activity assays performed with the extracts of aerial and root parts of *E. viviparum* are presented in Table 2. For the OxHLIA assay, it is given the extract concentration required to protect 50% of the erythrocytes from the haemolysis induced by the hydrophilic free radical initiator AAPH for 30 and 60 min. In addition, IC_{50} values are given at two Δt, as natural extracts contain a wide range of antioxidant compounds capable of offering protection at different time periods (Lockowandt et al., 2019). For the TBARS assay, it is given the extract concentration required to provide 50% of antioxidant activity, in other words, the dose at which the extracts can avoid the formation of malondialdehyde through the H donation to lipid radicals. Hence, in both assays, the lower the IC_{50}, the higher the antioxidant activity of the tested extracts.

As observed in Table 2, the antioxidant activity of the root extract was higher than that of the aerial part of *E. viviparum*. In TBARS, the IC_{50} value achieved with root extract (14.9 ± 0.3 μg mL^{-1}) was just 2.8-fold higher than that of trolox (5.4 ± 0.3 μg mL^{-1}), while the aerial part extract was 4.7-fold less effective than this synthetic antioxidant. However, it is interesting to note that trolox is a pure antioxidant compound, while plant extracts are complex mixtures of different phytochemicals with or without bioactive properties. In OxHLIA, 68 ± 3 μg mL^{-1} and 141 ± 6 μg mL^{-1} of root extract were needed to protect 50% of the erythrocyte population for 30 min and 60 min, respectively. This extract was 2.6% more effective than the one of the aerial part at both given Δt. Comparing with trolox, it can also be concluded that the *E. viviparum* extracts were more effective at 60 min than at 30 min. These results can be linked to the higher content of bioactive phenolic compounds, such as rosmarinic and caffeoylquinic acids, detected in the root of the studied endangered plant (Table 1), since polyphenols have the capacity to donate H^+ and e^- to free radicals, stabilizing them and giving rise to a quite stable phenolic radical (Heim et al., 2002).

To the best of authors' knowledge, this is the first study reporting the *in vitro* antioxidant activity of *E. viviparum* extracts, but there are reports with other *Eryngium* spp. including *E. serbicum* L. (Vukic et al., 2018), *E. bourgatii* (Cádiz-Gurrea et al., 2013), and *E. maritimum* (Mejri et al., 2017).

3.2.2. Cytotoxic activity

The results of the cytotoxic activity of *E. viviparum* extracts against four human tumour cells and a non-tumour porcine liver cell culture are displayed in Table 2. These are given as GI_{50} values, which correspond to the extract dose needed to inhibit 50% of cell growth. Hepatocellular carcinoma HepG2 was the most sensitive tumour cell line to both *E. viviparum* extracts (GI_{50} = 239 μg mL^{-1}). The root extract was also cytotoxic to the cell lines NCI-H460 (non-small cell lung carcinoma) and MCF-7 (breast adenocarcinoma), with GI_{50} values of 305 ± 10 μg mL^{-1} and 350 ± 12 μg mL^{-1}, respectively. On the other hand, none of the extracts was cytotoxic to HeLa cells (cervical carcinoma) or hepatotoxic to the normal PLP2 cells at the tested concentrations (GI_{50} > 400 μg mL^{-1}). Ellipticine, a potent antineoplastic agent used as positive control, revealed significantly higher cytotoxicity to the human tumour cells than *E. viviparum* extracts, but also to the non-tumour cell line. Once again, these results may be linked to the higher level of bioactive polyphenols detected in the root (Table 1).

Extracts or isolates from *Eryngium* spp. have been described to have cytotoxic activity against tumour cells (Wang et al., 2012). Monks et al.

Table 2
Antioxidant and cytotoxic activities of *E. viviparum* extracts and positive controls.

	Root	Aerial part	Positive control	Statistics[x]	
Antioxidant activity (IC_{50}, μg/mL)			Trolox	Homoscedasticity	*p*-value
TBARS	14.9 ± 0.3 [b]	25.6 ± 0.2 [a]	5.4 ± 0.3 [c]	0.896	< 0.001
OxHLIA, Δt = 30 min	68 ± 3 [b]	178 ± 3 [a]	8.1 ± 0.3 [c]	0.239	< 0.001
OxHLIA, Δt = 60 min	141 ± 6 [b]	371 ± 7 [a]	20.6 ± 0.9 [c]	0.358	< 0.001
Cytotoxic activity (GI_{50}, μg/mL)			Ellipticine		
NCI-H460 (lung cancer)	305 ± 10	> 400	1.03 ± 0.09	0.119	< 0.001
MCF-7 (breast carcinoma)	350 ± 12	> 400	0.91 ± 0.04	0.117	< 0.001
HeLa (cervical carcinoma)	> 400	> 400	1.91 ± 0.06	–	–
HepG2 (hepatocellular carcinoma)	239 ± 7 [a]	239 ± 2 [a]	1.1 ± 0.2 [b]	0.144	< 0.001
Hepatotoxicity (GI_{50}, μg/mL)					
PLP2 (liver primary culture)	> 400	> 400	3.2 ± 0.7	–	–

[x] Statistically significant differences ($p < 0.05$) between three or two variables were assessed by a one-way ANOVA, using the Tukey's HSD test for homoscedastic samples ($p > 0.05$), or by a Student's *t*-test, respectively.

Table 3
Antimicrobial activity of *E. viviparum* extracts and positive controls.

	Root		Aerial part		Positive control			
Antibacterial activity					Streptomycin		Ampicillin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. cereus	1	2	2	4	0.1	0.2	0.25	0.4
S. aureus	4	8	4	8	0.04	0.1	0.25	0.45
L. monocytogenes	2	4	2	4	0.2	0.3	0.4	0.5
E. coli	1	2	2	4	0.2	0.3	0.4	0.5
E. cloacae	2	4	2	4	0.2	0.3	0.25	0.5
S. typhimurium	2	4	0.5	1	0.2	0.3	0.75	1.2
Antifungal activity					Ketoconazole		Bifonazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. fumigatus	1	2	8	16	0.25	0.5	0.15	0.2
A. ochraceus	1	2	4	8	0.2	0.5	0.1	0.2
A. niger	4	8	8	16	0.2	0.5	0.15	0.2
P. funiculosum	0.5	1	2	4	0.2	0.5	0.2	0.25
P. ochrochloron	1	2	4	8	2.5	3.5	0.2	0.25
P. verrucosum	0.5	1	2	4	0.2	0.3	0.1	0.2

MIC: minimal inhibitory concentration (mg mL^{-1}); MBC: minimal bactericidal concentration (mg mL^{-1}); MFC: minimum fungicidal concentration (mg mL^{-1}).

(2002) described *E. ebracteatum* leaf and root organic extracts and *E. nudicale* aerial part aqueous extracts as having cytotoxic effects to NCI-H460. Paşayeva et al. (2017) described *E. creticum* aqueous extracts and *E. billardieri* methanolic extracts as capable of inhibiting MCF-7 cell growth (Paşayeva et al., 2017). Furthermore, Yurdakök and Baydan (2013) also reported cytotoxicity of *E. kotschyi* and *E. maritimum* aqueous extracts from aerial and root parts against HepG2 cells (Yurdakök and Baydan, 2013).

3.2.3. Antimicrobial activity

The results of the antimicrobial activity of *E. viviparum* extracts are presented in Table 3. In general, the Gram-negative bacteria were more sensitive to the tested extracts. *Bacillus cereus* and *E. coli* were the most susceptible bacteria to the root extract (MIC = 1 mg mL^{-1} and MBC = 2 mg mL^{-1}), which in general gave the best results. *Salmonella typhimurium* was the only microorganism to have greater sensitivity to the extract of aerial part and the one presenting the lowest MICs (0.5 mg mL^{-1}) and MBCs (1 mg mL^{-1}); interestingly, the MIC obtained for this enteric pathogen was lower than that of the positive control ampicillin (0.75 mg mL^{-1}). For *S. aureus* and L. *monocytogenes*, the MICs and MBCs obtained with the root extract were the same as those obtained with the aerial part.

The root extract also provided the best antifungal activity (Table 3), with MICs and MBCs between 0.5–4 mg mL^{-1} and 1–8 mg mL^{-1}, respectively. *A. niger* and *A. fumigatus* were the most resistant fungi to the tested *E. viviparum* extracts. In turn, *P. funiculosum* and *P. verrucosum* var. *cyclopium* were the most susceptible to the tested extracts, being required 0.5 mg mL^{-1} and 1 mg mL^{-1} of root extract and 2 mg mL^{-1} and 4 mg mL^{-1} of aerial part extract to inhibit or kill these two microorganisms. Interestingly, the root extract was more effective than ketoconazole in inhibiting and killing *P. ochrochloron*. As mentioned above, caffeic acid derivatives have many biological actives, including antimicrobial effects (Nabavi et al., 2015; Nunes et al., 2015), and could be linked to the observed effect.

Extracts of *Eryngium* spp. have shown a broad antimicrobial activity against a number of bacteria and fungi (Erdem et al., 2015). *E. maritimum, E. planum* and *E. campestre* hydroethanolic extracts (Thiem et al., 2010) and the apolar fraction of a methanol-chloroform-water extract of *E. maritimum* (Meot-Duros et al., 2008) revealed antibacterial activity against *B. cereus* and *S. aureus* and antifungal activity against *A. niger*. Methanolic and chloroform extracts of *E. palmatum* leaves and roots also showed activity against *S. aureus* and *E. coli* (Marčetić et al., 2014). In addition, fractions of methanolic extracts obtained from *E. caeruleum* aerial parts were effective against *E. coli*, *S. typhimurium*, *A. fumigatus*, and *A. niger* (Sadiq et al., 2016). However, to the best of the authors' knowledge, this is the first report on *Eryngium* extracts with antibacterial activity against *Enterobacter* and *Listeria* strain and antifungal activity against *Penicillium* genus.

4. Conclusion

In this study, the *in vitro* cultured *E. viviparum* plant was characterized as an interesting source of phenolic acids, particularly rosmarinic and *trans* 3-*O*-caffeoylquinic acids, which were particularly abundant in the root. Flavonoids were quantified just in the aerial part. The root extract showed the highest antioxidant activity, especially in the TBARS assay, and cytotoxicity to the NCI-H460 (non-small cell lung carcinoma), MCF-7 (breast adenocarcinoma), and HepG2 (hepatocellular carcinoma) cell lines. On the other hand, none of the extracts was cytotoxic to non-tumour primary PLP2 cells up to the concentration of 400 µg/mL. In general, the root extract also provided better antimicrobial activity than the aerial part extract. *Bacillus cereus* and *E. coli* were the most susceptible bacteria to this extract, while *P. funiculosum* and *P. verrucosum* var. *cyclopium* were the most susceptible fungi. Interestingly, the root extract was more effective than ketoconazole against *P. ochrochloron*, whereas *S. typhimurium* was the only microorganism to have greater sensitivity to the extract of the aerial part, with a MIC lower than that of ampicillin. Therefore, this study promotes the interest of conserving *E. viviparum* given its content in phenolic acids with antioxidant and antimicrobial properties, which can be exploited by different industrial sectors interested in bio-based ingredients.

CRediT authorship contribution statement

Manuel Ayuso: Methodology, Investigation, Formal analysis, Writing - original draft. **José Pinela:** Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Maria Inês Dias:** Methodology, Investigation. **Lillian Barros:** Conceptualization, Methodology, Formal analysis, Project administration, Writing - original draft, Writing - review & editing. **Marija Ivanov:** Methodology, Investigation. **Ricardo C. Calhelha:** Methodology. **Marina Soković:** Methodology, Investigation. **Pablo Ramil-Rego:** Funding acquisition, Project administration. **M. Esther Barreal:** Conceptualization, Investigation. **Pedro Pablo Gallego:** Conceptualization, Funding acquisition, Project administration, Writing - review & editing. **Isabel C.F.R. Ferreira:** Conceptualization, Methodology, Formal analysis, Funding acquisition, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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General discussion

General discussion

The present PhD thesis was aimed to explore new ways for the *ex situ* conservation and valorisation of endangered plants, particularly *Eryngium viviparum.*

As has been outlined in the general introduction, some strategies for the *in situ* conservation of plants has been applied to protect the endangered *Eryngium viviparum* species (Magnanon et al. 2012). However, the facts and data evidenced that this strategy has not had the expected success, since in less than 10 years (1997-2007) the classification of *E. viviparum* change from "vulnerable" to "endangered" due to the loss of subpopulations, decrease in the number of individuals and reduction of the area and quality of their habitat. Therefore, the current situation of the *E. viviparum* is critical and other *ex situ* conservation strategies such as *in vitro* culture (Ellstrand and Elam 1993; Romero et al. 2004; Serrano et al. 2019; Rascle et al. 2019).

The design of *ex situ* conservation strategies needs to consider the genetic diversity of the original populations. Thus, although the mother plant material used for of *ex situ* conservation can be diverse (living plants, tissue, vegetative propagules, pollen, etc.), seeds are the best option. Seeds allow to maintain the native genetic diversity and their collection from natural habitats has less impact than the removal of other plant materials (Roberts 1991; Fay 1992; Menges 2000). In this sense, our hypothesis was that *Eryngium viviparum* could be micropropagated from seeds, opening a new strategy for its *ex situ* conservation. However, we faced three unexpected problems related with the use of seeds as starting plant material for micropropagation: i) the high seed surface contamination; ii) the poor rate of seed germination and iii) the limited number of seeds available, due to strict policies of conservation as an endangered plant.

In the first chapter (*Computer-Assisted Recovery of Threatened Plants: Keys for Breaking Seed Dormancy of Eryngium viviparum*), a deep germination study of *E. viviparum* seeds is described. Due to the little information available on this species, the selection of the variables to be studied in the experimental design was based on

data and results published from plants of the same family (Apiaceae) and when available, from the same genus.

According to literature, no disinfection or soft sterilization procedures are necessary to successfully germinate Apiaceae species (Walmsley and Davy, 1997; Vandelook et al., 2008; Thiem et al., 2013). However, *E. viviparum* seeds revealed severe contamination by fungi during germination and none were able to germinate in preliminary experiments. The application of sulphuric acid, a method used to sterilize seeds heavily contaminated with fungi (Latchs and Christensen 1985; Siegel et al. 1987), achieved the total elimination of contamination in *E. viviparum* seeds. Additionally, seeds from *Eryngium* spp. have shown low (10-20%) germination rate (Mozumder and Hossain, 2013; Necajeva and Ievinsh, 2013; Thiem et al., 2013) and in the specific case of *Eryngium viviparum* populations from France and Spain (Magnanon et al., 2012), also low germination percentages (10 to 40%) were reported. Our preliminary results agreed with all those results, ranging from seed germination from 0% to 27%.

Seeds from Apiaceae family have a low germination rate due to: i) a high number of non-viable seeds; ii) the presence of morphological dormancy (seeds with underdeveloped embryos); iii) the fact that seeds may have an additional physiological component of dormancy (Ojala 1985; Baskin and Baskin 2014). Our results confirmed the high percentage of non-viable seeds percentage (62.5%) presenting embryoless (25.5% EL) and empty morphologies (37% EM). These results supported the hypothesis that the poor germination percentage of *E. viviparum* seeds can be explained as a consequence of the high percentage of non-viable seeds due to insect infestation and self-pollinated umbels (Flemion and Henrickson 1949; Ojala 1985; Zhang et al. 2019). The second cause of low germination in Apiaceae is the presence of underdeveloped embryos at the moment of dispersal (Martin 1946; Vandelook et al. 2008; Wolkis et al. 2020). The embryo needs to grow up to a critical length before germination. This delay in seed germination due to the underdeveloped and differentiated embryo is called morphology dormancy (Nikolaeva 1977; Baskin and Baskin 2014). Our results showed a high percentage of underdeveloped embryo seeds (30.4%) suggesting

that *E. viviparum* also showed MD, accord with other *Eryngium* sp. (Necajeva and Ievinsh 2013). The third cause for low germination rates is the presence of an additional physiological component of dormancy, which delays germination (Baskin and Baskin 2004). Thus, some Apiaceae species manifest both morphological (MD) and physiological (PD) dormancy, also denominate as morphophysiological dormancy (MPD). MPD seeds need specific treatments and a longer period for germinating than MD seeds (Baskin and Baskin 2014). Cold and warm stratification used in this work were effective for break MPD dormancy and allowing embryo to grow in other Apiaceae species. (Vandelook et al. 2008; Mozumder and Hossain 2013; Necajeva and Ievinsh 2013).

Neural networks tools were used to model and decipher the key factors of the treatments tested. The model pinpointed the following factors: time of incubation (20 weeks), low warm stratification (4 weeks), continuous high-temperature 24ºC and 1 mg L^{-1} GA_3 during incubation procedure are strongly recommended for successful seed germination.

As described above, micropropagation of wild plants from natural seeds germinated and cultured *in vitro* has been proposed as promising technique for *ex situ* conservation of *Eryngium viviparum* (Bañares et al., 2004; González-Benito and Martín, 2011), but due to the limited number of seeds, the poor rate of seed germination and the lack of *in vitro* culture procedures, it has not been implement to date in *E. viviparum*. However, micropropagation enables mass plant propagation and germplasm storage, thus preserving a representative genetic diversity, which constitutes a reliable methodology for plant conservation (Coelho et al. 2020). Moreover, micropropagation procedure is a justified priority in plants as *E. viviparum*, which present a low number of fragmented populations with a decreasing trend, and a limited geography range, such as *E. viviparum*. Thus, *ex situ* maintenance of threatening species is essential in these critical situations, as a guarantee to protect plants and the new specimens for reintroduction in damaged or new habitats (Cochrane et al. 2007).

The second objective of this PhD Thesis was to design a micropropagation protocol for *E. viviparum*, which is described in the second chapter of the results (*In vitro culture of the endangered plant Eryngium viviparum as a dual strategy for its ex situ conservation and source of bioactive compounds*).

Micropropagation protocols included 5 steps: i) Selection of mother plant; ii) culture establishment; iii) propagation; iv) rooting and v) acclimatization (Debergh et al. 1992; George et al. 2008). Seedlings *in vitro* germinated were used as initial plant material (step 1). The results showed that all seedlings (100%) were established successfully in MS medium supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IBA. No contamination was observed in the media, since all seedlings established proceeded from *in vitro* germination. For multiplication, (George et al. 2008) cytokinins are essential for a successful propagation since promote lateral shoots formation. A factorial design to study the effect of 9 treatments with two cytokinins combinations (BAP and KIN) at 3 levels (0, 1 and 2 mg L^{-1}; 3 BAP levels x 3 KIN levels; named T1-T9) were tested. The results suggest that high concentration of BAP (2 mg L^{-1}) combined with any concentrations of KIN promoted the highest new formed shoot number, in agreement with previous reports in *E. foeitidum* (Gayatri et al. 2006; Chandrika et al. 2011). Finally, although in long-term cultures high concentrations of cytokinins may produce very small and/or hyperhydric shoots (Debergh et al. 1992; George et al. 2008), this kind of shoots not were found in our study (at least until the eighth subculture). For rooting (step 4), some authors suggested the use of auxins such as IBA for successful root growth (Makunga et al. 2003; Sharma et al. 2004; Karuppusamy 2009); nevertheless, in Apiaceae family and also in some *Eryngium* genus, the rooting was promoted according to sucrose and salt concentration on the media. (Thiem et al. 2013; Kikowska et al. 2014, 2016). Our results support these reports, demonstrating rooting was influenced by 2% of sucrose and half-strength MS media salts. Acclimatization of *in vitro*-grown plants to *ex vitro* conditions is the last stage in micropropagation procedure. Our protocol allowed acclimatizing 96% of the rooted plantlets, which agree with previous results described in other *Eryngium* genus.

As described in detail in the general introduction, plant *in vitro* culture technology constitutes a great biotechnological tool for the study of plant secondary metabolism, 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). In fact, the high number of plants micropropagated in this study allows a continuous supply of material for test their phytochemical potential.

The aerial plant material obtained from micropropagation experiments were employed to determine the total phenolic and flavonoid content, and the free-radical scavenging activity. Our results showed that the combination of both cytokinins (BAP and KIN) at high concentrations (2 mg L^{-1}) elicited the production and accumulation of phenolic compounds in aerial parts. Some reports (Treutter 2010; Dias et al. 2016) suggested that cytokinins enhance the cinnamic acid biosynthesis pathway generating different precursors of phenolic compounds. Additionally, the high BAP concentrations also promoted a large antioxidant activity, which may be related with several phenolic acids such as chlorogenic, caffeic and rosmarinic acids, with antioxidant activity, that were founded in high concentrations in several species of *Eryngium* (Le Claire et al. 2005; Wang et al. 2012; Thiem et al. 2013; Kikowska et al. 2014).

Thus, the third results chapter (*Phenolic composition and biological activities of the in vitro cultured endangered Eryngium viviparum J. Gay*) describe, for the first time, all experiments carried out to determine the phenolic compound composition and the antioxidant, antimicrobial, and cytotoxic activities of the aerial and root parts of *Eryngium viviparum* micropropagated plants. Hydroethanolic extracts of both organs were analysed regarding phenolic compounds composition (phenolic acids and flavonoids) together with their related bioactivities. Mainly phenolic acids were determined, as already described by the available literature (Kikowska et al. 2014; Vukic et al. 2018), being the majority derived from caffeic, rosmarinic, and caffeoylquinic acids. Additionally, a glucuronide-conjugated of tectorigenin was the identified flavonoid, namely tectorigenin-*O*-glucuronide. This isoflavone is a potent antiplatelet compound

than acetylsalicylic acid. It has also been shown to exert anti-proliferative effects on tumour cells (Thelen et al. 2005).

For the determination of antioxidant activity, TBARS and OxHLIA assays were performed. In both experiments, roots extracts have stronger activity than aerial parts. These results can be linked to the higher content of bioactive phenolic compounds, such as rosmarinic and caffeoylquinic acids, detected in the roots of the studied endangered plant compared with the concentration of the aerial parts. Concerning cytotoxic activity, extracts or isolates from *Eryngium* genus have been reported for their activity against tumour cells (Wang et al. 2012; Yurdakök and Baydan 2013; Monks et al. 2019). In our study, four human tumour cells and a non-tumour porcine liver cell culture were tested. None of both root and aerial parts extracts were hepatotoxic to non-tumour primary PLP2. HepG2 (hepatocellular carcinoma) was the most sensitive cell line to both extracts, in agreement with others *Eryngium* previously reported as *E. kotschyi*, and *E. maritimum* (Yurdakök and Baydan 2013). Finally, concerning to the antimicrobial activity assay, Gram-negative bacteria were the most sensitive to *E. viviparum* extracts. *Bacillus cereus* and *E. coli* were the most susceptible bacteria to the root extract and *Salmonella typhimurium* to the aerial parts. The most vulnerables fungus to the tested extracts were *P. funiculosum* and *P. verrucosum* var. cyclopium. The results can be linked to caffeic acid derivatives present in the samples, which have a high antimicrobial effect (Nabavi et al. 2015; Nunes et al. 2015). Other *Eryngium* species have been reported for their broad antimicrobial activity against several bacteria and fungi (Meot-Duros et al. 2008; Erdem et al. 2015). However, this is the first time that *Eryngium* sp. extracts showed antibacterial activity against *Enterobacter* and *Listeria* strains, and antifungal activity against *Penicillium* genus.

In summary, this PhD Thesis, presented as a compendium of publications, achieved the implementation of an *ex situ* conservation strategy for *Eryngium viviparum*, describing the causes and consequences of its low germination, establishing a new and specific protocol for *in vitro* propagation, and promoting its value as a medicinal plant due to the presence of several bioactive compounds, which could be interesting to exploit as bio-based ingredients.

Conclusions

Conclusions

This study hypothesis was that "*Eryngium viviparum* could be micropropagated from seeds, opening a new strategy for its ex situ conservation, and producing enough plant material to evaluate its phytochemical and therapeutical potential, without damaging the natural populations".

To evaluate the initial hypothesis, three specific objectives were described and their main conclusions were:

1.- This study confirms that *Eryngium viviparum* seeds present morphological dormancy (MD) and revealed a physiological component of dormancy (PD), which reduce their germination. To improve this ratio in MPD seeds, stimulating the embryo development, the next germination procedure is proposed: long incubation time (20 weeks) combined with low warm stratification (4 weeks), incubation at continuous high temperature (24ºC) on a media supplemented with 1 mg L^{-1} GA_3. Under those conditions, *E. viviparum* seeds reached 89-100% of germination.

2.- A very simple, efficient and successful micropropagation protocol was established, allowing high rates of shoot multiplication (5.5), root formation (100%) and plantlets acclimatization (96%). The acclimatized plants were re-introduced in the natural habitats constituting an effective *ex situ* conservation strategy. Additionally, this study provides a novel insight about the phenolic content of *in vitro* cultured *Eryngium viviparum* plants and its antioxidant activity, unravelling its phytochemical potential for industrial applications.

3.- The phenolic profile and bioactive potential of *Eryngium viviparum* were reported for the first time. Rosmarinic and trans 3-O-caffeoylquinic acids were the most abundant phenolic acids in the roots of *E. viviparum* plantlets cultured *in vitro*. Antioxidant, antibacterial, antifungal, and cytotoxic activities in aerial and root extracts were also described.

In summary, this study demonstrated that biotechnological and artificial intelligence procedures are helpful tools to conserve threatened plants with

potential medicinal properties, generating new specimens to increase their wild populations and to exploit as potential bioactive ingredient sources for different industrial sectors.

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