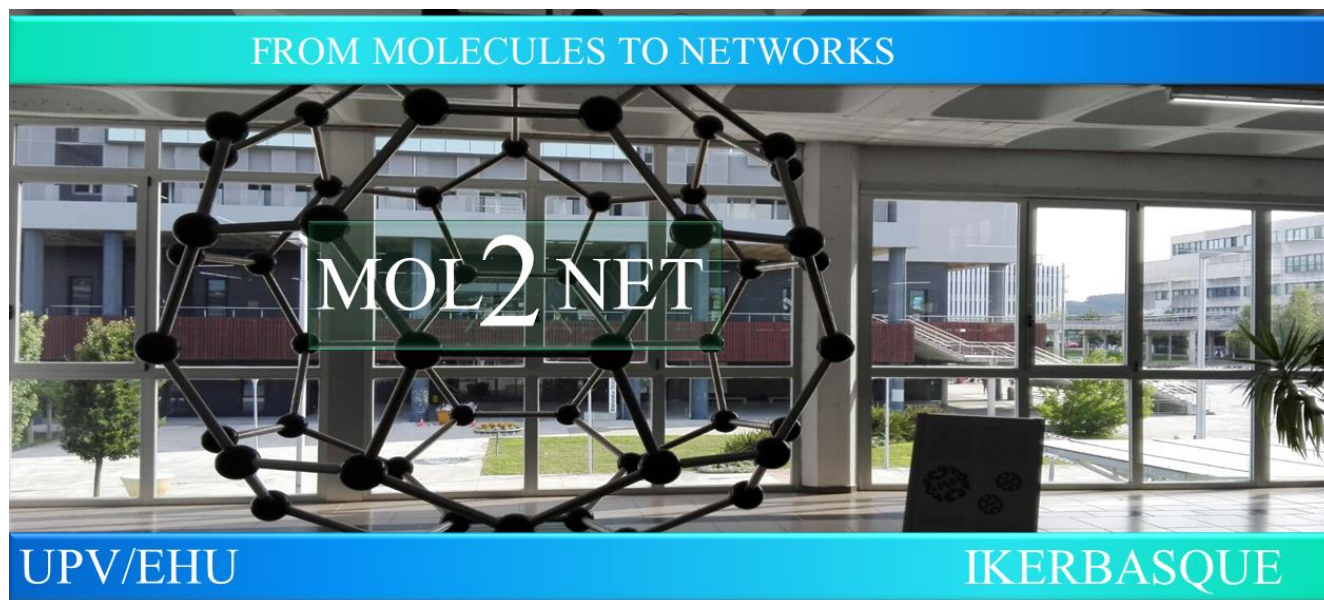




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Thymol elicitation during *in vitro* regeneration of axillary bud explants from a *Thymus piperella* L. commercial hybrid

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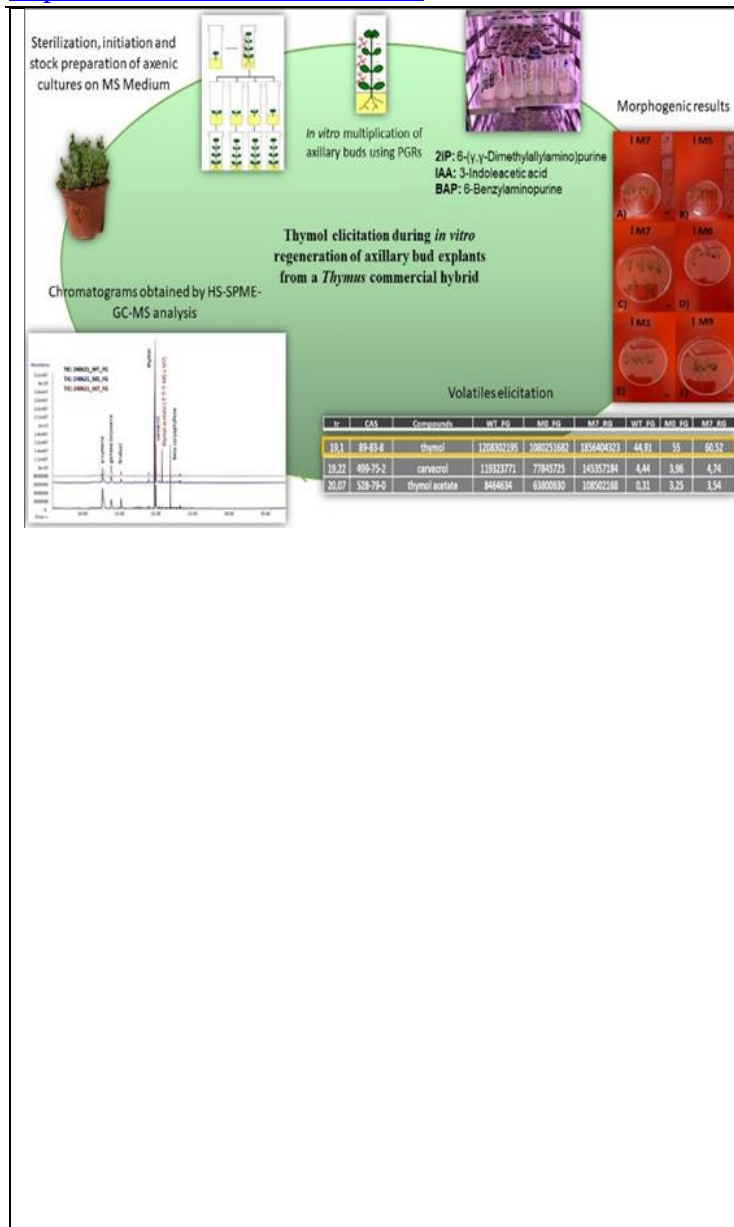
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Graphical Abstract

Abstract.

Species of the genus Thymus L. (Lamiaceae) are of great interest as medicinal plants, as well as in the extracts and food technology industries due to the richness in bioactive specialized metabolites. Specifically, Iberian endemic species such as Th. piperella L., have a wide range of traditional uses in this sense. However, the micropropagation and biosynthesis of high-added value compounds under in vitro culture conditions has not been



studied for this species and its commercial hybrids yet. Therefore, the main objective of this work was to develop a micropropagation protocol of a popularly used commercial hybrid of *Thymus*. The results obtained showed a good capacity of adaptation and initiation of *in vitro* culture after the sterilization treatment applied. In addition, the different plant developmental parameters measured in the multiplication phase showed significantly higher values in Murashige and Skoog (MS) medium supplemented with 0.013 mg/L 2-isopentenyladenine (2iP), or without plant growth regulators, plus 30 g/L sucrose and solidified with 5.5 g/L Plant Agar when compared to the other treatments applied. Finally, the test performed on the content of volatile compounds by HS-SPME-GC/MS revealed that there was a remarkable increase of thymol in the *in vitro* cultivated plants when compared to the samples from the wild-type plants. Therefore, the results obtained in the present work could be the basis for the development of future studies on the elicitation of this compound by *in vitro* culture systems with this hybrid, or other plant materials from *Thymus piperella* chemotypes.

Introduction

The genus *Thymus* L. is represented by around 220 described species in the world (Khajuria et al., 2020), and 36 subspecies (Kosakowska et al., 2020). Most of them have a great record of traditional uses as medicinal plants, spices, and aromatic species with interests in the industry of plant extracts due to their richness in volatile compounds (terpenes), which have been related to the antioxidant, antifungal, and antibacterial properties, among many others, of this plants' extracts (Ballester, 2016). Very well-known examples are the popular thyme (*Th. vulgaris* L.), and marjoram or white thyme (*Th. mastichina* L.). Locally, it also remarkable the widespread use of various Iberian endemic species such as "tomillo salsero" (*Th. zygis* L.), "pebrella" (*Th. piperella* L.) or "cantueso" (*Th. moroderi* Pau ex Martínez). These species have been subject of domestication programs, and selected genotypes and hybrids are being also produced in nurseries to be used locally as a medicinal plants and spices (Morales et al., 2011). Plant tissue culture techniques are employed to produce big amounts of plants to be commercially exploited (Hussain et al., 2012). These methods have been extensively applied to species of the genus *Thymus*, including both, *Th. vulgaris* (Leal et al., 2017), and *Th. piperella* (Sáez et al., 1994). These biotechnological approaches can be also used to enhance the biosynthesis *in vitro* of specialized

metabolites such as volatile compounds with high added value (thymol, carvacrol, camphor, etc.). Nonetheless, the application of the *in vitro* culture techniques for elicitation of these compounds in locally important species from the Iberian Peninsula, and their commercial hybrids, is very limited. For these reasons, the aim of this work was to develop an *in vitro* multiplication protocol of a commercial hybrid of *Th. piperella*, as well as to characterize and compare the phytochemical profile of both *in vitro* cultured plantlets, and wild-type plants.

Materials and Methods

The mother plants (wild-type, WP) were acquired in a local market in Valencia, and kept in outdoor conditions until the beginning of the experiments, in April 2021. For surface sterilization of the plant material, selected stems were cut and immersed in 70% ethanol solution (v/v) for 30 seconds. Then, the plant material was transferred to a solution of sodium hypochlorite (active chlorine content, 40 g/L) at 7% (v/v) for 20 minutes. Next, 3 washes in sterile distilled water were carried out before sowing onto initiation medium. Explants employed to initiate the cultures consisted in axillary buds of around 0.2-0.3 cm. All culture media (for initiation, and multiplication) were based in Murashige and Skoog (MS) salts with vitamins (Murashige and Skoog, 1962) plus 30 g/L sucrose, and solidified with 5.5 g/L Plant Agar. The culture medium for initiation consisted in the above stated composition without plant growth regulators (PGRs). Environmental conditions for culture initiation were, as follows: first cultivation in the dark for 10 days, followed by cultivation for 30 more days in a 16-hours photoperiod, both at constant temperature of 21±1 °C. For multiplication experiments, 11 treatments were applied based in different types and concentrations of PGRs (6-Benzylaminopurine, BAP; 6-(γ,γ -Dimethylallylamino)purine, 2iP; 3-Indoleacetic acid; IAA) in the basal medium above described. In this stage, the effects of PGRs on the explants was assessed after 30 days of *in vitro* culture conditions in a 16-hours photoperiod, at constant temperature of 21±1 °C as well. The experiment was set up using ten explants per replicate, and 3 replicates per PGR treatment under the above mentioned culture conditions. The parameters of plant development studied included the number of new shoots, leaves, nodes, branches, and roots generated, as well as the shoot and root elongation (in cm). Finally, the chemical profile of both, WT and *in vitro* produced plants in media showing better performance in developmental parameters (M7 and M0) was studied, and compared. For this experiment, plant samples were dried in the dark at room temperature conditions (around 22 °C). After drying, a sample of 0.02 g from the plant material was subjected to Solid Phase Micro-Extraction (SPME) with subsequent analysis by Gas Chromatography coupled to Mass Spectrometry (GC/MS) using the Headspace (HS) technique. Identification of compounds was done by using the spectra library present in the software NIST Chemistry WebBook. Then, 16 compounds were quantified using 14 terpene standards purchased from Sigma Aldrich (Barcelona, Spain). The chemical analyses were aimed to determine qualitatively the presence of these compounds, and to quantify them by percentage of area (% area). For the statistical analysis of the results, the free software "Infostat" (National University of Córdoba, Argentina) was used. Analysis of the results was carried out by ANOVA and significant differences were revealed by using the Fisher's least significant difference test (LSD) at a confidence level of 99% ($\alpha = 0.01$).

Results and Discussion

The sterilization procedure here applied resulted in an explant survival higher than 90%, thus showing its suitability for this *Thymus* hybrid initiation under *in vitro* culture conditions. Initiation was successful as all axenic cultures could restore their growth, and elongated after the 40-days culture period. These plantlets served as starting materials (new axillary buds) for further multiplication experiments. The results obtained after 30 days of *in vitro* multiplication using 11 combinations of PGRs showed that treatment M7 (0.013 mg/L 2iP) gave significantly better results ($p < 0,01$) in terms of plant development (higher number of newly formed leaves, nodes, branches, and root length) when compared to the other treatments. Also, M0 (without PGRs) offered suboptimal results for these parameters, and, together, treatments M7 and M0 gave significant better results in shoot elongation and number of newly formed roots (not statistically different among them) when compared to the other 9 multiplication treatments ($p < 0,01$). These morphogenic results are comparable to the multiplication performance obtained for other *Thymus* species (El Ansari et al., 2019; Leal et al., 2017). 2iP has also shown better multiplication performance under *in vitro* conditions in some Lamiaceae species such as *Th. vulgaris* (El Ansari et al., 2019) or *Sideritis leucantha* Cav. subsp. *leucantha* (Juan-Vicedo et al., 2021) when compared to other cytokinins such as BAP. BAP is widely used in plant regeneration *in vitro*, but often performs lower rates in terms of new shoots per explant, and shoot elongation in *Thymus* species (Leal et al., 2017). This suggests a sensitivity of these plants to the PGRs exogenously applied, as we observed in this *Th. piperella* hybrid. The chemical analyses revealed that major compounds (in % area) for all plant materials analyzed (WT, and *in vitro* cultured plants) were thymol, p-cymene, gamma-terpinene, carvacrol, beta caryophyllene, and linalool which supports the hybrid origin of this specimen, between *Th. vulgaris* and *Th. piperella*. Also, elicitation of thymol on *in vitro* cultured plants was observed with respect to the WT plant. *In vitro* culture conditions can modify the biosynthesis of specialized metabolites in plants (Bekircan et al., 2018; Hussain et al., 2012). In this study, we observed that treatments M0 and M7 remarkably increased the production of thymol with respect to WT samples (% area: WT=44,91%; M0=55,00%; M7=60,52%). Given the importance of thymol in several fields of applications, the results obtained in the present work highlight the suitability of the *in vitro* culture techniques not only for the industrial production of *Thymus*, but also for the elicitation of the biosynthesis of this high-added value compound.

Conclusions

A protocol of in vitro culture, and thymol elicitation for a commercial Th. piperella hybrid is proposed here. Immersion of stems from WT plants in 70% ethanol (v/v) for 30 seconds, followed by immersion in sodium hypochlorite (active chlorine content, 40 g/L) at 7% (v/v) for 20 minutes resulted in more than 90% of axenic explants during the initiation phase in medium MS with vitamins plus 30 g/L sucrose, and 5.5 g/L Plant Agar. Cultures were kept at constant temperature of 21±1 °C in a 16-hours photoperiod. The treatment consisting of 0.013 mg/L 2iP, added to the above mentioned medium, and cultured in the same environmental conditions, showed significant higher values of plant development in terms of number of newly produced shoots, nodes and leaves, as well as elongation, and rooting of the plantlets grown in vitro, compared to the other 10 media compositions studied. Also, the analysis of volatile compounds carried out suggested that this PGR treatment under in vitro conditions increases thymol biosynthesis.

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