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Effects of copper sulphate ($CuSO_4$) elicitation on the chemical constitution of volatile compounds and the *in vitro* development of Basil

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ABSTRACT

Basil produces important compounds for the industrial manufacture of medicines, cosmetics, and food that are obtained by conventional production techniques. *In vitro* cultivation has contributed to the increasing reliability of the production, and efficient isolation, of target compounds independent of seasonal factors. We sought to assess the effects of copper sulfate on seedling growth and the elicitation of chemical constituents of the essential oil of basil leaves cultivated *in vitro*. Basil seedlings were grown in Murashige and Skoog (MS) medium enriched with 25 and 75 μM $CuSO_4$, as well as in control treatments. Essential oils obtained from the hydrodistillation of dried leaves were subjected to GC–MS analysis. The GC–MS and PCA analyses indicated the presence of two predominant volatile groups: Phenylpropanoids (77.81%) in the 25 μM $CuSO_4$ treatment, with eugenol and methyl eugenol as major components, with industrial and pharmacological importance; and Monoterpenes (69.72%), in the 75 μM$CuSO_4$ treatment and control, subdivided into monoterpene hydrocarbons (19.36%) and oxygenated monoterpenes (50.36%), with 1,8 cineole and linalool as major components.MS medium enriched with 25 μM $CuSO_4$ resulted in seedlings with longer and more numerous leaves, which would be important for oil extraction. That Cu concentration probably affected the phenylpropanoid metabolic pathway, which is triggered mainly under plant stress situations.

1. Introduction

Biotechnological tools now allow the genetic modification of cells, tissues, organs, or *in vitro* cultivated plants as alternatives to plant breeding programs and secondary metabolite production (Gonçalves and Romano, 2013; Alvarez, 2014). Tissue culture techniques appear promising for obtaining bioactive compounds, as they produce high biomass yields and phytochemical production (Morais et al., 2012; Miralpeix et al., 2013). Obtaining secondary metabolites through tissue culture rather than conventional cultivation is advantageous because there are no seasonal restrictions to production. Tissue culture also provides production reliability and predictability, as well as rapid and efficient isolation of target compounds (Ahsan et al., 2013; Gonçalves and Romano, 2013), making the commercial production of secondary metabolites very attractive (Zhao et al., 2005; Alvarez, 2014).

Basil (*Ocimum basilicum* L.) is a medicinal herb originally grown in India, Africa, and South Asia. This aromatic plant is popularly used as a culinary seasoning, as an active ingredient in traditional medicine, and for increasing the shelf-life of food (Bertoli et al., 2013). Basil is also highly valued for its pharmaceutical characteristics, due to the antioxidant properties of essential oils extracted from its leaves (Bais et al., 2002). Scientific studies have demonstrated that *Ocimum* sp. produces considerable amounts of secondary metabolites, with linalool as the major component of its essential oil (EO), followed by *epi*-α-cadinol, α-bergamotene, γ-cadinene, germacrene-D, and camphora (Hussain et al., 2008). These compounds are largely produced using conventional cultivation techniques, with wide variations in their yields and composition (Hussain et al., 2008).

Elicitors are molecules that can alter plant metabolic pathways when added in low concentrations to the culture medium, and increase the production of secondary metabolites (Chen et al., 2001; Namdeo, 2007; Baenas et al., 2014); they have been employed to increase the quantities of secondary metabolic compounds produced *in vitro* (Vasconsuelo and Boland, 2007; Baenas et al., 2014). Diverse types of elicitors have been used to treat medicinal plant cultures, including: methyl jasmonate, rosmarinic acid, salicylic acid, phytonutrients,

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microorganisms, yeast extracts, salts, and metals such as copper sulfate (Gundlach et al., 1992; Georgiev et al., 2007; Baenas et al., 2014; Kikowska et al., 2015; Giri and Zaheer, 2016).

Copper is an essential micronutrient for plant growth, being a co-factor in diverse physiological processes such as photosynthesis, cell wall metabolism and lignification, and as a constituent of superoxide dismutase (SOD) (Yruela, 2005). Copper is also present in catalytic enzymes, aids in protein transport, and acts as transcription flag for some genes (Marschner, 2011). Perotti et al. (2010) added 175 μM $CuSO_4$ to *in vitro* tissue cultures of *Alternanthera philoxeroides* and observed a 60% increase in betacyanin production. There have been no published studies examining the use of copper as elicitor for the common green *O. basilicum,* although Złotek et al. (2016) found that jasmonic acid (at 100 μM) elicited high yields of linalool, eugenol, and limonene in *O. basilicum,* and also increased methyl eugenol production when added to the culture medium at 1 μM.

As such, we expected that the *in vitro* cultivation of *Ocimum* sp. with copper sulfate as an elicitor would increase the numbers of leaves and promote biomass production without negatively affecting plant growth while simultaneously increasing the amounts of secondary metabolic compounds present in its essential oil (Zhao et al., 2005; Alvarez, 2014). This would assure continuous, large-scale productions of those compounds, avoid seasonal effects on productivity, and allow production predictability (Ahsan et al., 2013; Gonçalves and Romano, 2013). We therefore assessed the effects of copper sulfate elicitation on basil seedling growth and the chemical constitution of the essential oils of *O. basilicum* plants (leaves) cultivated *in vitro*.

2. Materials and methods

2.1. Plant material and seed asepsis

All experiments were conducted in the Molecular Biology and Plant Culture Tissue laboratories of the Paranaense University (UNIPAR). Green basil seeds (Feltrin®; batch number 443767) for micropropagation were purchased from a local market. The selected seeds were treated with 70% ethanol for 2 min and then immersed in 2% sodium hypochlorite solution for 15 min, with agitation. The seeds were subsequently rinsed four consecutive times with sterile distilled water.

2.2. In vitro Conditions

The culture media used contained MS medium (Murashige and Skoog, 1962) enriched with 30 g L^{-1} sucrose, 0.1 mg L^{-1} benzylaminopurine (BAP), and 0.2 mg L^{-1} naphthaleneacetic acid (NAA), at pH 5.8, and hardened with 6.5 g L^{-1} agar (Kasvi). Based on studies by Perotti et al. (2010), $CuSO_4$ concentrations of 0 μM (T0, as the control), 25 μM (T1), and 75 μM (T2) were added to the culture medium. The medium was then transferred to 350 ml flasks and autoclaved at 121 °C for 20 min. The aseptic seeds were then sown into clear glass flasks that were closed with clear plastic lids and sealed with polyvinylpyrrolidone film (PVP). All treatments were subjected to identical conditions, being maintained in a growth chamber at 25 ± 2 °C under a 24 h photoperiod with an irradiance level of 3600 lx provided by Empalux® 10–20 w–6400 K cool white fluorescent lamps. A completely randomized design was used in the experiment, with 3 treatments and 6 replicates, with 25 flasks per replicate and 4 seeds per flask.

2.3. Isolation of the essential oil

Eighty-three days after elicitation, total of 120 flasks per treatment (T0, T1, and T2) were separated for essential oil analysis. The *O. basilicum* seedlings were removed from the flasks and their leaves were dried at 25 ± 2 °C and subsequently submitted to hydro-distillation in a modified Clevenger-type apparatus for 3h. After extraction, the essential oils were dried over anhydrous sodium sulfate and stored until GC/MS analysis.

2.4. Chemical characterization of the essential oils

Samples of the essential oils obtained from different levels of elicitation (0, 25, and 75 μM $CuSO_4$) were subjected to GC/MS analysis, using an Agilent 19091S-433 gas chromatograph coupled to an Agilent 19091J-433 mass spectrometer. The capillary column was HP-5MS 5% (30 m × 0.250 mm × 0.25 μm). Helium was used as carrier gas at a flow rate of 1 mL min^{-1} and a constant pressure of 80 kPa. The temperatures of the injector and detector were 260 °C and 280 °C respectively. The essential oil samples were diluted with dichloromethane (1:10); the injection volume was 1.0 μL, using a split mode (1:2). The temperatures of the transfer line, ions source and quadrupole were 280 °C, 230 °C and 150 °C respectively. The column temperature was initially programmed at 40 °C, with heating at 4 °C $min^{-1}$to reach a final temperature of 300 °C. The detection system was MS with the Scan mode in a mass/charge range of 40–550 m/z, with a solvent delay of 3 min. The retention indices (RI) of the components of the essential oils were determined based on the alkane series C9–C44. The identifications of individual components were made by comparisons of their mass spectra with the mass spectra in WILEY 275 libraries, and by the means of their retention indices, as compared with those in the literature (Adams, 2012).

2.5. Cluster analysis

Multivariate exploratory hierarchical clustering and principal component analyzes were performed, allowing for the conjoint evaluation of all of the variables analyzed. The hierarchical clustering technique interconnects the samples by their associations, producing a dendrogram or tree in which similar samples are grouped together according to the variables chosen (Moita Neto and Moita et al., 1998). Euclidean distances were used as the dissimilarity metric was used to represent the straight-line distances between the centroids of each cluster of chemical compounds identified in the essential oils. The Unweighted Pair Group Method with Arithmetic Averages (UPGMA) was adopted for clustering the compounds. The results are presented in a dendrogram form, which facilitates cluster characterization.

2.5.1. Principal component analysis

Principal component analysis captures the greatest amount of original information contained in the *p* variables. For each treatment, the chemical compounds and their quantities (%) (Table 5) were plotted on Excel spreadsheets. Compounds present in only trace amounts (t) and those that were not identified (ni) were not considered in our analyses. Data were transformed through orthogonal rotation into latent variables named principal components, which are linear combinations of original variables, created from the eigenvalues of the data covariance matrix (Hair et al., 2005). Kaiser criterion was used to choose the principal components. Eigenvalues preserve relevant information when greater than unity. Analyses were performed using Statistica 13.3 software (Statsoft, 2017).

2.6. In vitro culture evaluation

Evaluations were performed at three different times during seedling cultivation (54, 69, and 83 days after elicitation). These evaluation periods were determined according to morphological development of the seedlings, considering the maximum period of seedling survival in MS medium. Observations were made for germination (%), callus formation, abnormal seedlings, and plant oxidation (%). Fungi and bacteria were considered contaminants. Seedlings were considered abnormal when displaying morphological alterations such as twisted leaves, twisted stems, or other deformations (Brasil, 2009).

A 3 × 3 factorial experimental design was used with three