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Elicited *Teucrium chamaedrys* cell cultures produce high amounts of teucrioside, but not the hepatotoxic *neo*-clerodane diterpenoids

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ABSTRACT

Teucrium chamaedrys, one of the most common and investigated species of the genus *Teucrium*, has been used for centuries in traditional medicine for many purposes. Its phytochemical components comprise, among others, phenylethanoid glycosides (PGs) and *neo*-clerodane diterpenoids. Several reports have demonstrated a wide range of beneficial biological and pharmacological activities of the phenylethanoid components, while the diterpenes were shown to be strongly hepatotoxic. In this work, *in vitro* cultures were established from leaf explants of *T. chamaedrys*. Both solid (callus) and liquid (cell suspension) cultures maintained the capacity to produce PGs, with teucrioside (TS) representing the most abundant one. Cell suspensions had a lower TS content than that found in leaf extracts, but higher than that of calli. An NMR-based metabolomics approach was used to compare the product profile of intact plants vs. cell suspension cultures, and results showed that *neo*-clerodane diterpenes, present in the intact plant, were not detected in cell cultures. Several elicitors were supplied to cell cultures with the aim of increasing TS production, and elicitation was tested at different growth phases and by exposing cells for different periods. Methyl jasmonate and fungal mycelia from *Trichoderma viridae* and *Fusarium moniliforme* were able to significantly increase TS production if supplied at the early-exponential growth phase for 24 h. Based on the proposed link between proline and the phenylpropanoid pathways, proline accumulation in cell cultures was followed throughout a 14-day culture period, showing that it strictly reflected that of TS production. Moreover, exogenously supplied proline, and its analogue hydroxyproline, turned out to be very effective in increasing teucrioside production.

1. Introduction

The genus *Teucrium*, belonging to the Lamiaceae family, is represented by about 100 species, distributed throughout the world, but mainly abounding in the northern temperate and subtropical regions of the Eastern Hemisphere. Various species of this genus have been used for over 2000 years in traditional medicine for their diuretic, diaphoretic, tonic, antispasmodic and cholagogic properties (Ulubelen et al., 2000); more recently, the interest towards *Teucrium* species has increased, due to a pronounced anticancer activity demonstrated for plant extracts and isolated compounds of these plants (Stankovic et al., 2011). Moreover, extracts from some species were shown to potentiate the cytotoxic and proapoptotic effects of anticancer drugs vincristine, vinblastine and doxorubicin, against a panel of cancer cell lines (Rajabalian, 2008).

One of the most common and highly investigated species in the genus is *Teucrium chamaedrys*, commonly called germander. It is native to Europe, and used in the treatment of digestive and respiratory disorders, abscesses, gout (Stankovic et al., 2010), and, externally, as an astringent infusion on the gums and in the treatment of wounds (Chiej, 1984). Hydroalcoholic extracts of this plant are currently used as approved substances in the preparation of flavored wines, bitters and liqueurs. Phytochemical constituents comprise diterpenes (in particular *neo*-clerodane diterpenoids), monoterpenes and other classes of compounds including saponins, glycosides (iridoids and phenylethanoids), and flavonoids (Bedir et al., 2003; Pacifico et al., 2009). *neo*-Clerodane diterpenoids constitute a large group of natural toxic compounds, and in particular two of them, teucrin A and teuchamaedryn A, are considered to be responsible for several cases of hepatotoxicity associated to the use of this plant in human beings (Gori et al., 2011). Phenylethanoid glycosides (PGs) are the main phenolic components in *Teucrium* species, and several reports have demonstrated the wide range of biological and pharmacological activities of these compounds

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(Korkina et al., 2007). Among them, teucrioside (TS), the first L-lyxose-containing PG found in nature (Gross et al., 1988), has only been detected in *T. chamaedrys*, and is, therefore, used as marker compound for the characterization of this species (Avula et al., 2003). In a recent report, Pacifico et al. (2009) evaluated the antioxidant properties of pure metabolites, as well as of crude extracts of this plant, and found that PGs were responsible for a strong radical scavenging capacity. Similar results were obtained by Stankovic et al. (2010), who reported, for extracts of this plant, an antioxidant activity very similar to that of *Ginkgo biloba* L. and *Camellia sinensis* L., considered as reference plants for their very high antioxidant activity. The beneficial effects of PGs have, indeed, been traditionally explained in terms of the prevention of free radical-associated and transition metal-mediated cell and tissue damage.

Plant cell cultures represent a valid alternative to whole plants for the production of high-value secondary metabolites (Bourgaud et al., 2001; Ramachandra Rao and Ravishankar, 2002). Nevertheless, besides some undoubted advantages of this technology, mainly related to the higher rate of metabolism, the shorter biosynthetic cycle compared to differentiated tissues, and the possibility of monitoring production under controlled conditions all year round, strategies aimed at increasing the yield of the required metabolites is often necessary to solve the problem of the low productivity associated to the undifferentiated state of plant cell cultures. These strategies include treatment with elicitors and signal compounds such as jasmonates, chitosan and fungal elicitors (Yukimune et al., 1996). In fact, phenylpropanoid biosynthesis is among the most frequently observed metabolic activity that is induced upon treatment of plant tissues or cultured cells with elicitors (Barber et al., 2000). Elicitation has, in some cases, been obtained by exogenous application of the jasmonic acid (JA) derivative methyl jasmonate (MJ), which can induce the expression of genes responsive to wounding and pathogen attack, and which encode for defense-related compounds (Creelman and Mullet, 1997), including protective secondary metabolites (Blechert et al., 1995; Horvath and Chua, 1996). Gadzovska et al. (2007) demonstrated a strong activation of the phenylpropanoid pathway in *Hypericum perforatum* by treatment with MJ, while JA induced the production of ajmalicine and catharanthine in *Catharanthus roseus* (Vazquez-Flota and De Luca, 1998), and rosmarinic acid and shikonin in *Lithospermum erythrorhizon* (Mizukami et al., 1992; Yazaki et al., 1997). The production of several types of compounds has also been successfully enhanced by treating cell cultures with fungal elicitors, such as catharanthine in *C. roseus* (Zhao et al., 2001), cryptotanshinone in *Salvia miltiorrhiza* (Chen and Chen, 2000), oleandrin in *Nerium oleander* (Ibrahim et al., 2007), and saponin in *Panax ginseng* (Lu et al., 2001).

Phenolic metabolites in plants are efficiently produced through an alternative mode linking proline synthesis with the oxidative pentose phosphate pathway (Shetty, 2004; Yang and Shetty, 1998). In the model proposed by Kwok and Shetty (1998), a cellular redox cycle linking proline and its precursor pyrolline-5-carboxylate can stimulate the pentose phosphate, shikimate and phenylpropanoid pathways, and ultimately lead to an increase in phenolics synthesis. In shoot cultures of oregano the exogenous application of proline did, in fact, elicit phenolic metabolism (Lattanzio et al., 2009), and in shoot cultures of thyme an increase in total phenolics and rosmarinic acid contents was observed after supplying the proline analog hydroxyproline (Kwok and Shetty, 1998). Such evidence has led to attribute an elicitor role to the "multifunctional" amino acid proline.

In view of the interesting pharmacological and biological activities of PGs, the present work was performed to establish cell cultures of *T. chamaedrys*, and to investigate their capacity for producing PGs, in particular TS, a fact which has not been previously reported. An NMR-based metabolomics approach was used to compare the product profile of intact plants vs. cell suspension cultures. In order to investigate the possibility of enhancing PG production, elicitation of cell cultures by chitosan, fungal elicitors, MJ, proline and hydroxyproline was also performed.

2. Results and discussion

2.1. Cell culture establishment and growth rate

After 4 weeks of culture on MS medium supplemented with 0.5 mg/l 2,4-D and 0.2 mg/l kinetin, leaf explants of *T. chamaedrys* developed a green callus, which, after several subcultures, became friable and was used to initiate suspension cultures. After 10 subcultures, the growth of cell suspensions was measured in terms of both fresh weight and cell number (Fig. 1). The former displayed an initial lag phase of four-five days, followed by an exponential phase of eight days, and then a stationary phase (Fig. 1A). Maximum fresh biomass was obtained between days 13 and 16. Specific growth rate (μ), doubling time (dt), and growth index (GI) were determined during three consecutive subculture cycles; no significant differences were observed between subcultures (average values $\mu = 0.083 \pm 0.006\ d^{-1}$, dt = 8.37 ± 0.63 d, GI = 4.24 ± 0.31), suggesting that a stable line was obtained (Bourgaud et al., 2001). During the exponential growth phase, an increase in cell number, i.e., cell division activity, dominated the capacity of the cultures to grow, as evident from the more rapid increase in cell number compared to fresh weight (Fig. 1B). Cell viability remained high during one subculture, ranging from 95% in the first 10 days to 78% at the end of the culture cycle (Fig. 1B).

The growth parameters of *Teucrium* cell culture turned out to be similar to those of *Buddleja cordata* green cell cultures producing phenylethanoids such as verbascoside and related compounds such as linarin and hydroxycinnamic acids (Estrada-Zúñiga et al., 2009).

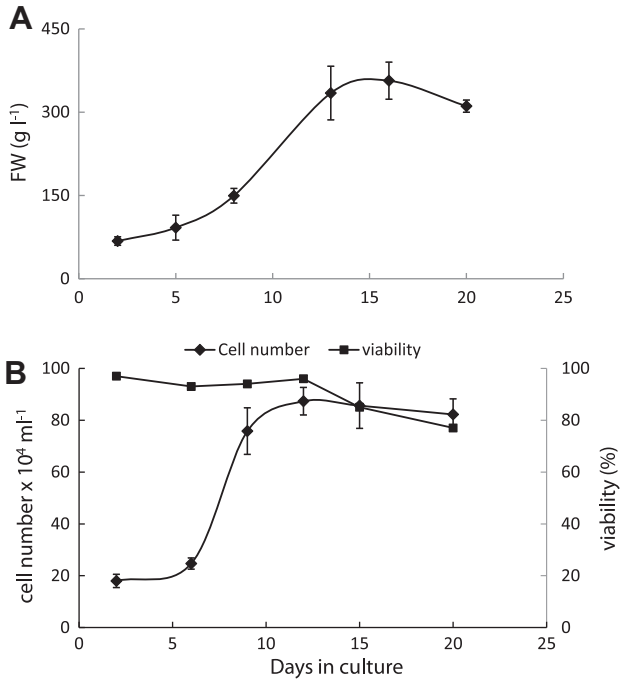

Fig. 1. Growth and viability of *T. chamaedrys* cell suspension culture expressed as FW (A) and cell number (B). Data are the means ± SD of triplicate measurements, and determinations were repeated for three subsequent subculture cycles.