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Production of caffeic, chlorogenic and rosmarinic acids in plants and suspension cultures of *Glechoma hederacea*

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ABSTRACT

Glechoma hederacea L. (Lamiaceae) is a perennial plant, which is distributed widely in Europe, Asia and America. Important anti-oxidant compounds are caffeic acid esters like rosmarinic acid (RA) and chlorogenic acid (CA). Phenylalanine ammonia-lyase (PAL) and rosmarinic acid synthase (RAS, 4-coumaroyl-CoA:hydroxyphenyllactic acid hydroxycinnamoyltransferase) contribute to the formation of RA. Our aim in this study was to follow the accumulation of RA, CA and caffeic acid in a suspension culture of *G. hederacea*. Growth, medium and secondary metabolism parameters were determined during a culture period of 14 days. The maximal PAL activity was observed on day 5 and the maximal RAS activity on day 8. The RA content was exceedingly high and reached 25.9% of the dry mass on day 7. Caffeic acid and CA contents remained rather low. Furthermore, the presence of RA, CA and caffeic acid and the expression patterns of RAS and hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase (HST), an important enzyme of monolignol formation, in leaves, flowers, stems and roots of naturally grown *G. hederacea* were assessed. The expression of RAS and HST genes was detectable in all organs except roots. Flowers accumulated 12.5% RA in their dry mass, leaves, stems and roots about 1%. CA was highest in leaves (2.0%), while it was at 1.6% in flowers, 1.3% in stems and almost undetectable in roots. The caffeic acid content remained at or below 0.4% of the dry weight in all organs.
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1. Introduction

Glechoma hederacea L. (Lamiaceae), commonly known as ground ivy, is a perennial plant, which is distributed widely in Europe, Asia and America. It has been used in folk medicine for centuries for the treatment of various diseases e.g. cholelithiasis, urolithiasis, inflammation, cold, asthma and dropsy (Kim et al., 2011a). *G. hederacea* extracts were shown to have depigmenting effects on skin (Ha et al., 2011; Qiao et al., 2012) and might be useful to control macrophage-mediated inflammatory diseases (An et al., 2006). They also displayed significant anti-oxidant capacity (Milovanovic et al., 2010).

G. hederacea belongs to the sub-family Nepetoideae of the Lamiaceae, which is known for its accumulation of rosmarinic acid (RA). The presence of chlorogenic acid (CA) in Lamiaceae is much more widespread (Litvinenko et al., 1975; Pedersen, 2000; Petersen et al., 2009). However, both compounds also occur in species of other families throughout the plant kingdom (Clifford, 1999; Petersen et al., 2009). RA, an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid (Scarpati and Oriente, 1958), and CA, an ester of caffeic acid and quinic acid (Panizzi et al., 1955), are the main active phenolic compounds in *G. hederacea*. It is supposed that plants use these compounds as defense against pathogens, herbivores and as UV protectant (Clé et al., 2008; Petersen et al., 2009; Sánchez-Campillo et al., 2009). In addition, RA-derivatives with methylation of the carboxyl group and/or varying substitution patterns at the phenylpropenoid moiety and benzyl-4′-hydroxybenzoyl-3′-*O*-β-D-glucopyranoside have been isolated from *G. hederacea* var. *longituba* (Kim et al., 2011a). Other secondary metabolites identified in *Glechoma* are flavonoids, lignans, norlignans, tropane alkaloids (hederacins), sesquiterpenes, sesquiterpene lactones, triterpenoids, essential oil and lectins (Kikuchi et al., 2008; Kim et al., 2011b; Kumarasamy et al., 2003; Mockute et al., 2005; Wang et al., 2003; Zhu et al., 2013; Zieba, 1973a,b).

RA has shown numerous biological and pharmacological activities, e.g. inhibition of the attachment of *Herpes simplex* virus 1 (Astani et al., 2012) or anti-bacterial and anti-inflammatory properties (Parnham and Kesselring, 1985). Recently, Bulgakov et al. (2012) published a review summarizing the various biological activities of RA.

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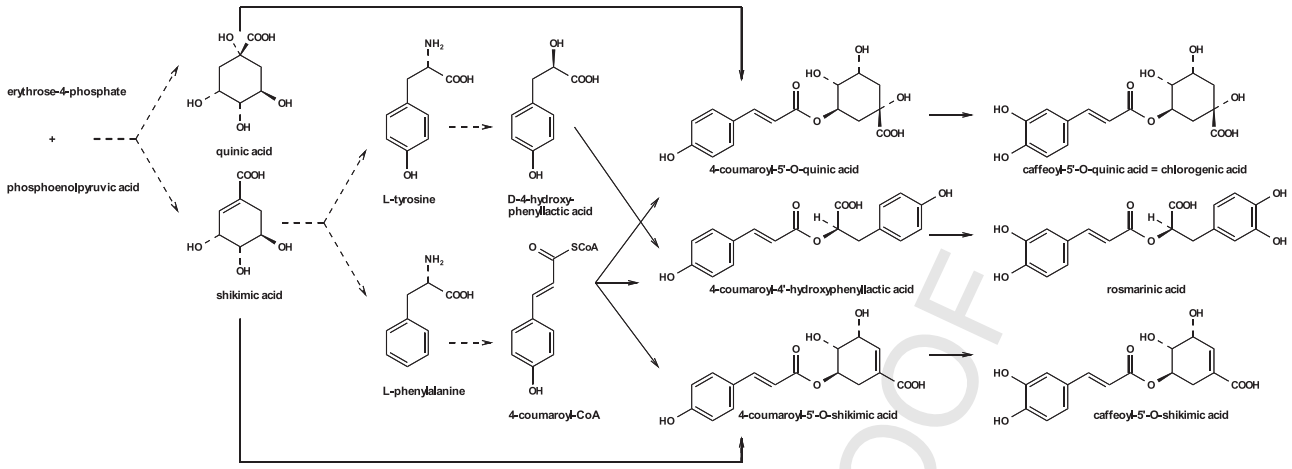

Fig. 1. Scheme of rosmarinic acid, chlorogenic acid and caffeoyl shikimic acid formation.

In plants, CA performs anti-oxidant functions. After inhibiting CA accumulation in tobacco, an accelerated cell death in mature leaves and an elevated lipid peroxidation level were observed (Tamagnone et al., 1998). Another function of CA is in UV-protection (Clé et al., 2008). In the apoplastic space CA works together with peroxidases in hydrogen peroxide scavenging (Sakihama et al., 2002; Takahama et al., 1999). In vacuoles, CA can complex other secondary metabolites (e.g. tropane alkaloids) and thus be part of the driving force to transport metabolites into the vacuole (Pardo Torre et al., 2013; Waldhauser and Baumann, 1996).

The precursor for the formation of RA and CA (Fig. 1) is L-phenylalanine formed by the shikimic acid pathway, which also delivers shikimic and quinic acid as well as L-tyrosine. L-Phenylalanine is transformed to 4-coumaroyl-CoA by the well-known enzymes of the general phenylpropanoid pathway starting with phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.24). Rosmarinic acid synthase (RAS, hydroxycinnamoyl-CoA:hydroxyphenyllactic acid hydroxycinnamoyltransferase, E.C. 2.3.1.140) is the characteristic enzyme of RA biosynthesis that transfers the 4-coumaroyl moiety from 4-coumaroyl-CoA to the aliphatic hydroxyl group of 4-hydroxyphenyllactic acid (pHPL) and forms an ester (Petersen and Alfermann, 1988; Petersen, 1991), which is afterwards hydroxylated by cytochrome P450 monooxygenase activities at the aromatic positions 3 and 3′ to RA (Petersen, 1997). Similarly hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (HQT, E.C. 2.3.1.99) transfers the 4-coumaroyl moiety to quinic acid forming 4-coumaroylquinate (Stöckigt and Zenk, 1974), which is then hydroxylated to CA (Franke et al., 2002; Heller and Kühnl, 1985; Kühnl et al., 1987). Both, RAS and HQT are members of the BAHD acyltransferase superfamily (Berger et al., 2006; Hoffmann et al., 2003; St.Pierre et al., 2000). A third hydroxycinnamoyltransferase preferably transfers the hydroxycinnamoyl moiety to shikimic acid (hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase, HST, E.C. 2.3.1.133) and thus forms 4-coumaroyl-/caffeoylshikimate, which are supposed to be important intermediates in the formation of monolignols (Hoffmann et al., 2004; Niggeweg et al., 2004). The discrimination of the acceptor substrates between HST and HQT often is not strict.

Sander (2010) isolated two cDNA sequences each of HST, RAS and an additional hydroxycinnamoyltransferase (HCT) from *G. hederacea* (see Section 2). Unfortunately, a cDNA sequence encoding a HQT has not yet been detected in *G. hederacea* or other Lamiaceae species.

The aim of our study was to show the presence of caffeic, chlorogenic and rosmarinic acid in *G. hederacea* cell suspension cultures and to characterize the time course of their accumulation. Furthermore we investigated the caffeic, CA and RA contents in flowers, leaves, stems and roots of *G. hederacea* and assessed the expression levels of HST and RAS genes via semi-quantitative PCR in these organs.

2. Results and discussion

2.1. Characterization of a G. hederacea suspension culture

In order to elucidate the capacity of a suspension culture of *G. hederacea* to accumulate caffeic acid and its derivatives rosmarinic and chlorogenic acid (RA, CA) we established a callus and a suspension culture of this species and characterized the suspension culture during a culture period of two weeks in CB-medium with 2% sucrose (CB2; Petersen and Alfermann, 1988).

The fr. weight of the cells increased from 2.5 g per flask (50 g/l) on day 0 to 10.9 g per flask (218 g/l) on day 7 (Fig. 2A). The dry weight also increased continuously until a maximal value of 520 mg/flask (10.4 g/l) was observed on day 6 of the cultivation period (Fig. 2A). After having reached the maximum values, fr. and dry weight decreased slowly until the end of the observation period due to cell death and lysis.

The pH-value of the cell-free medium increased slowly from pH 5.4 on day 0 to pH 7.5 on day 11 (Fig. 2B). The electrical conductivity decreased continuously until day 6, but increased again until the end to approximately the initial value (Fig. 2B). The decrease reflects the uptake of ions from the medium, while the following increase can be explained by cell death with concomitant release of intracellular compounds to the medium. A similar behavior was observed in a suspension culture of *Melissa officinalis* (Weitzel, 2009). The sugar content of the medium (measured by the refractive index) decreased from the beginning of the culture period and showed a very steep drop from day 4 to day 5. From that time point the sugar level remained approximately at the same low level until the end of the cultivation period (Fig. 2B). The depletion of the carbohydrate source and the cessation of ion uptake correlate with the end of the cells' growth phase.

Lyophilized cells were extracted with 70% ethanol and analyzed for their contents of caffeic acid, RA and CA by HPLC. In contrast to RA, the caffeic acid and CA levels were rather low throughout the culture period. The highest CA content of 0.4% of the cell dry weight was measured on day 1. The CA content remained at low levels throughout the culture period (Fig. 2C). A suppression of CA accumulation in the dark was reported for e.g. suspension cultures of *Nicotiana plumbaginifolia* (Gillet et al., 1999) or *Coffea arabica*

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