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Water deficit stress fluctuates expression profiles of *4Cl*, *C3H*, *COMT*, *CVOMT* and *EOMT* genes involved in the biosynthetic pathway of volatile phenylpropanoids alongside accumulation of methylchavicol and methyleugenol in different Iranian cultivars of basil

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ABSTRACT

Here, for the first time, the accumulation ratio of methylchavicol and methyleugenoland compounds together with the expression profiles of five critical genes (i.e., *4Cl*, *C3H*, *COMT*, *CVOMT* and *EOMT*) in three Iranian cultivars of basil were assessed under water deficit stress at flowering stage. The highest value of methylchavicol was detected for Cul. 3 under severe stress (*S3*; 7.695 μg/mg) alongside Cul. 2 under similar circumstances (*S3*; 4.133 μg/mg), while regarding Cul. 1, no detectable amounts were acquired. Considering methyleugenol, Cul. 3 (0.396 μg/mg; *S0*) followed by Cul. 1 (S3; 0.160 μg/mg) were the capable plant samples in producing some detectable amounts of methyleugenol. Apart from some expectations, all the genes under study exhibited also different transcription ratios under deficit stress. Our results, overall, demonstrated that the regulation of the above-mentioned genes and production of methychavicol and methyleugenol seems to be a cultivar- and drought stress-dependent mechanism.

1. Introduction

Basil (*Ocimum basilicum* L., *Lamiaceae* family), as an annual, ornamental, culinary, herbaceous and aromatic plant, is climatologically distributed profusely in the tropical and subtropical regions including Iran, and currently regarded as one of the globally most important medicinal plant, particularly, for the production of essential oil (Ekren et al., 2012). In fact, throughout history, the plant has been cultivated widely which is mainly attributed to its pivotal roles for treatment of broad spectrum of ailments like common headaches, cold and cough, ring worms, stomach-ache, sore throats, kidney malfunctions and hepatic disorders (Simon et al., 1999). Such or similar pharmaceutically important properties are argued to be rooted in a wide range of volatile aromatic essential oils (Kicel et al., 2005), which are biologically synthesized and stored in specialized anatomical structures known as “glandular trichomes”, located on the surface of the aerial parts of the plant (Gang et al., 2001).

Thus far, in excess of 140 different constituents have been recorded from the essential oil of *O. basilicum* (Hiltunen and Holm, 2006), depending upon the disparities in chemotypes, aroma, leaf and flower colors, growth and developmental phases of plant tissues, the geographical origin of the plants alongside environmental circumstances (Carović-Stanko et al., 2010; Ekren et al., 2012; Pirbalouti et al., 2013). Essential oils of *O. basilicum* are rich in phenylpropanes like eugenol, methyleugenol, chavicol, methylchavicol and some terpenoid compounds, which are contributed to the particular properties of many spices and herbs (Gang, 2005). Eugenol and chavicol are also found in significant amounts, 70–90% of the essential oil and the dry weight of clove buds, cinnamon leaves and in smaller amounts in nutmeg and pepper corns, which together widely used as culinary herb and fragrance supplies (Gang et al., 2001). These plant-based metabolites are important components of the defensive arsenal of plants, serve as signal

Abbreviations: ANOVA, analysis of variance; COMT, caffeoyl-CoA *O*-methyltransferase; CAD, cinnamoyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; C4H, cinnamate 4-hydroxylase; CAAT, coniferyl alchol acyl transferase; C3H, *p*-coumarate 3-hydroxylase; 4CL, 4-coumarate-CoA ligase; CoA, coenzyme A; CVOMT, chavicol *O*-methyltransferase; EGS, eugenol synthase; EOMT, eugenol *O*-methyltransferase; FC, field capacity (FC); GC–MS, gas chromatography–mass spectrophotometry; PAL, phenylalanine ammonia-lyase; Phe, phenylalanine; qRT-PCR, quantitative real-time PCR; RCBD, randomized complete block design; RWC, relative water content; RT, retention times; HCT, shikmate *O*-hydroxyl cinnamoyl transferase; SD, standard deviation

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molecules between plants and microbes and humans have made extensive use to further protect their plants and food stocks (Dixon et al., 2002). For example, eugenol serves as an antibacterial compound against the growth of many significant food-borne pathogenic bacteria, effective antifungal agent as well as a good nematocide at low dosages (Devi et al., 2010; Šimović et al., 2014). In contrast, the pleasant, fresh-smelling of methyleugenol is an important insect pollinator attractant of many floral scents, attracting pollinating in particular, being a female pheromone mimic for several fruit flies (Nishida, 2014). Thus, the uses of *O. basilicum* for therapeutic purposes and industrial importance can be owing to these compounds as a potential source of bioactive substances.

Many studies describing the biosynthesis of terpenoid constituents of herb essential oil, with proteins and genes involved in the pathway having been purified and cloned (Withers and Keasling, 2007). Surprisingly, the detailed biosynthetic pathways of the biosynthesis of eugenol, chavicol and their derivatives have not yet been established, in details. Breifly, the biochemical steps involved in the biosynthesis of the phenylpropanoids can be classified into several general stages (Baxter and Stewart, 2013; Dixon et al., 2002). The first committed step begins with the conversion of phenylalanine (Phe), an amino acid product of the shikimate pathway, to cinnamic acid via phenylalanine ammonia-lyase (PAL) (Dixon et al., 2002) (Fig. 1). By the next stage, by the activity of cinnamate 4-hydroxylase (C4H) as the second enzyme in the pathway, a cytochrome P450 monooxygenase, *t*- cinnamic acid is hydroxylated into 4- coumaric acid which is funneled into branched pathways (Dixon et al., 2002; Ehlting et al., 2006). As Fig. 1 is shown, in the one way, 4- coumaric acid which is then catalyzed to coenzyme A-thioester by 4- coumarate-CoA ligase (4CL), a member of the acyl adenylate forming enzyme, catalyzing the 3- hydroxylation and diversion of the carbon skeleton towards volatile phenylpropenes pathways (Gang et al., 2002). The initial steps of the pathway provide the basis for all subsequent branches and resulting metabolites in the phenylpropanoid pathway, are considered transcriptionally controlled regulatory steps. These reactions may involve the prior formation of the coenzyme A (CoA) esters, and it may also play the central role in regulating overall flux and channeling intermediates towards subsequent metabolic biosynthetic pathways in the phenylpropanoid network diverge (Vogt, 2010). Cinnamoyl-CoA reductase (CCR) catalyzes the reduction of cinnamoyl-CoA esters into cinnamaldehydes, which is considered to be the predominant role in the volatile phenylpropine-specific branches of the chavicol biosynthesis. Then, coniferyl alchol acyl transferase (CAAT) catalyzes final step in chavicol pathway by catalyzing the conversion of *p*-coumaryl-acetate into the chavicol biosynthesis. The final step in the biosynthesis of the methylchavicol, the conversion of chavicol to methylchavicol, is catalyzed by the enzyme chavicol *O*-methyltransferase (CVOMT). Also, for most plant species, the critical role of CCR in lignin biosynthetic pathway is the production of coniferaldehyde from feruloyl-CoA. Previous studies contributed to our current understanding of the role of cinnamoyl-CoA reductase and coniferyl alcohol acetyl transferase which are important enzymes directly upstream from chavicol production and which apparently expressed at very low levels in the breeding basil line (Rastogi et al., 2014). Inhibition of an upstream enzyme affects the downstream product several steps away, as at each step the efficiency of the enzymes may decrease due to reduced availability of substrates. The second direction proposes a route which *p*-coumarate 3-hydroxylase (C3H) catalyzes the production of caffeic acid from *p*- coumaric acid and direct the carbon flow from the shikimate pathway to the different branches that provide precursors for methyleugenol compound biosynthesis (Franke et al., 2002). The researchers suggested that the decrease of C3H activity may have led to the production of ester-linked glucosides, which can prevent a potentially toxic accumulation of the substrate by allowing it to be mobilized to the phloem (Coleman et al., 2008). caffeoyl-CoA *O*-methyltransferase (COMT), a member of the *O*-methyltransferase, methylates caffeoyl-CoA to feruloyl-CoA which is considered to be the critical step in the ability to transport of precursors into eugenol biosynthesis (Gang et al., 2001). However, the next steps in the production of eugenol after the formation of ferulic acid are not known, previous reports based on feeding incorporated of radioactive labeled precursors to whole leaf tissues suggested that eugenol is formed via an undefined mechanism, catalyzed by CCR, cinnamoyl alcohol dehydrogenase (CAD) and CAAT, of the monolignol precursor coniferyl alcohol, is also considered to be a regulatory step, as the precursors may be directly converted to the production of eugenol (Gang et al., 2002; Koeduka et al., 2006). However, other reports recently have proposed the addition of the methyl group to the 4-OH, by the enzyme eugenol *O*-methyltransferase (EOMT), is a pivotal last step in the formation of methyleugenol (Gang et al., 2002).

The water deficit stress is a critical abiotic stress which exerts a considerable influence on the plant physiology, productivity and specially the production of higher levels of secondary metabolites, including triterpenoids and phenylpropanoids (Selmar and Kleinwächter, 2013). Despite a number of investigations have been previously accomplished for basil in different research areas (Al-Kateb and Mottram, 2014; Ekren et al., 2012; Flanigan and Niemeyer, 2014; Gang et al., 2002; Gang et al., 2001; Moghaddam et al., 2014; Renu et al., 2014), according to the best of our knowledge, there is no investigation specifically focusing on the simultaneous assessment of the influence of different levels of water deficit stress on the expression profiling of the genes known to be expressed in the biosynthetic pathway of volatile phenylpropanoids as well as the possible production of the two important type of phenylpropanoid compounds known as methylchavicol and methyleugenoland in different cultivars of Basil. With this in mind, the current work was accordingly aimed to determine the production/accumulation ratio of methylchavicol and methyleugenoland compounds together with the expression profiles of five critical genes (i.e., *4Cl*, *C3H*, *COMT*, *CVOMT* and *EOMT*) in three Iranian cultivars of basil in response to three levels of water deficit stress (i.e., mild, moderate and severe) at flowering stage.

2. Materials and methods

2.1. Plant materials and cultivation conditions

In general, three Iranian cultivars of *O. basilicum*, originating from geographically two different areas of Iran [i.e., Amol (Cul. 2 and 3) and Jahrom (Cul. 1) cities] were employed as starting plant materials. The seeds of each cultivar were subjected first to sterilization process with submerging in 10% sodium hypochlorite for 5 min and immediately washed thrice using sterilized autoclaved water to remove disinfection liquid. Afterwards, the germination process of the seeds was undertaken in an isolated nursery located at the research greenhouse of Faculty of Agriculture, University of Tehran, Iran, with the beginning time of Mid-May, 2014. For each cultivar, three out of ten-day-old seedlings were transferred into individual pots containing sandy-loam soil and allowed to continue their growth under optimum circumstances for four additional weeks. In applying water deficit stress treatment, three different stress levels were adjusted first in relation to field capacity (FC), nominated then as *S1*: 75% FC, *S2*: 50% FC and *S3*: 25% FC and subsequently the healthy and uniform seedlings were transferred into each pot (Fig. 2). Notably, all the control plants were maintained under optimal irrigation conditions. The work was performed in a factorial scheme (3 × 4) based on a completely randomized design with two factors (i.e., three cultivars and four water-deficit stress treatments) and three replications. Sampling of the three cultivars was performed at full bloom stage (from May to June), after applying one month water scarcity stress treatment. The shoot samples of each treatment were harvested carefully and subsequently detached into two parts: the first one immediately frozen in liquid nitrogen and stored at −80 °C until further analyses (for gene expression assay), while the second one allowed to dry completely at 25–30 °C for GC–MS analysis.