Introduction

The geraniol-rich lemongrass (Cymbopogon flexuosus Nees ex Steud.) Wats mutant cv. GRL-1 was isolated from normal citral-rich cv. OD-19 during a chemo-genetical improvement program. GRL-1 is characterized by a high geraniol (G) content (~91%) compared to the normal citral-rich cv. OD-19 and other cultivars (Patra et al., 1997). The high level of G in mutant cv. GRL-1 might be due to the blockage of the NADP-geraniol dehydrogenase activity which otherwise in parent cv. OD-19 oxidizes G into geranial (citral) (Fig. 1). G is an acyclic monoterpenic alcohol which is frequently used in perfumery, soaps, detergents and cosmetics. G is also emitted from the flowers of many species, notably roses (Iijima et al., 2004), and it is a major constituent of palmarosa (C. martinii) essential oil (Dubey et al., 2000). It is also present in vegetative tissues of many herbs (Charles and Simon, 1992; Patra et al., 1997; Mallavarapu et al., 1998; Mockute and Bernotiene, 1999; Vieira et al., 2001; Sidibe et al., 2001).

In general, G is synthesized from geranyl diphosphate (GPP), the universal precursor of all monoterpenes (Croteau, 1987; Gershenzon and Croteau, 1993; McGarvey and Croteau, 1995; Wise and Croteau, 1999). However, in lemongrass mutant cv. GRL-1 we suggest at least three enzymatic activities to be involved in regulating the level of G.

(1) Either a phosphatase- or monoterpen synthase-based catalysis of GPP leading to G. Perez...
et al. (1980) have reported phosphatases which hydrolyze prenyldiphosphates (GPP; farnesyl diphosphate, FPP, and neryl diphosphate, NPP) to their corresponding alcohols such as geraniol, farnesol and nerol in citrus. In contrast, more recently geraniol synthase was reported to be involved in geraniol formation from GPP in basil, Cinnamomum and Perilla (Iijima et al., 2004; Yang et al., 2005; Michiho and Honda, 2007).

(2) Acetylation of G to geranyl acetate (GA) by a very active transacetylase. Shalit et al. (2003) have cloned and characterized the first acetyl-CoA acetyl transferase from rose petals which converts G into GA. However, transacetylase from Cymbopogon sp. could not be isolated.

(3) Finally, GA slowly hydrolyzes into G during leaf development. This reaction is catalyzed by an esterase referred to a geranyl acetate esterase (GAE). One such GAE catalyzing the biotransformation of GA into G has been reported previously from palamarosa (C. martinii) inflorescence (Dubey and Luthra, 2001; Dubey et al., 2003). Though, the level of G in the lemongrass mutant cv. GRL-1 is regulated by these three enzymatic steps, but GAE plays a crucial role in the formation of G during leaf development. In addition, different regulatory controls operating at organ, cellular, subcellular and enzyme/isoenzyme levels also play a role in controlling the level of GA and G (Dubey and Luthra, 2001; Dubey et al., 2003). Therefore, investigating these enzymatic steps is crucial to obtain a deeper understanding of the formation of G in lemongrass.

The aim of the present study was to examine the changes in the monoterpane (GA and G) content and composition and the enzymatic processes underlying these changes during leaf development in lemongrass mutant cv. GRL-1. The study revealed that the monoterpane (GA and G) content and composition markedly fluctuate during leaf development. In fact, the amount of GA in the immature (10 d) got completely converted into G; by the time all leaves reached maturation (50 d). Here we report as the enzyme GAE which is mainly involved in the conversion of GA to G. GAE is a developmentally regulated enzyme. During leaf development, the GAE activity has been found to be consistent with the monoterpane (GA and G) compositions substantiating its role in controlling and regulating the level of G. Moreover, the GAE isoenzyme patterns have also been in full agreement with the monoterpane composition during leaf development. Besides the role of GAE, the role of phosphatases in the formation of G from GPP has also been investigated at a preliminary level. For the present study, we choose lemongrass mutant cv. GRL-1 as it offers an excellent system to study the metabolism of G because in this mutant G does not further oxidize to citral.

Material and Methods

Plant material

Cymbopogon flexuosus (Ness ex Steud.) Wats mutant GRL-1 plants were grown in the Experimental Farm, Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. Leaves were harvested after 10 d of emergence and consequently at regular intervals of 10 d to study the monoterpane content and composition of the essential oils. In parallel, essential oils were also isolated to study the monoterpane content and composition of leaves at different positions in 50-day-old (fully expanded) tillers as the leaves from inside (1st) to outside (6th) of a tiller represent a gradient increase in leaf age. Moreover, to augment the developmental changes in the monoterpane compositions, 15-day-old leaves were cut into three equal parts, viz. basal (proximal), middle and apical (distal), and subjected to essential oil extraction. Being a monocot in nature, these three regions within the leaf represent the gradient increase in leaf age.

Essential oil extraction

For the isolation of essential oils, fresh leaf samples (~100 g) were collected, thoroughly washed...
under the tap, and cut into small pieces. Essential oil was isolated by steam-distillation technique using a mini-Clevenger apparatus. The volume of oil was measured and collected in small stopper tubes containing anhydrous sodium sulfate to remove traces of moisture.

**GC analysis**

The monoterpene compositions of the essential oils were analyzed by a Perkin Elmer 8500 gas chromatograph equipped with a flame ionization detector and a BP-1 (diethyl polysiloxane) column (30 m × 0.32 mm i.d., 0.25 μm film thicknesses). The temperature of the column was programmed from 60 °C to 220 °C at 5 °C min⁻¹ with the split ratio of 1:80. The carrier gas was nitrogen at a flow rate of 48.2 kPa. The G and GA peaks were identified by co-injecting authentic standards and quantified using a Varian integrator (model 4400).

**GAE enzyme extraction and assays**

GAE was extracted and assayed according to the procedure described by Dubey and Luthra (2001). GAE was extracted using 100 mM sodium phosphate (NaPi) buffer (pH 6.5) consisting of 50 mM sodium metabisulfite, 10 mM each of 2-mercaptoethanol and ascorbic acid, 0.25 M sucrose and 1 mM Na₂EDTA. Leaf samples (1 g) were ground by addition of insoluble PVPP (50% w/w) in extraction buffer (1:3 w/v). The homogenate was filtered through four layers of muslin cloth and centrifuged at 15,000 × g for 45 min. The supernatant collected was treated with purified amberlite XAD-4 resin (50% of the tissue weight) for 5 min at 4 °C to remove the endogenous terpenes. The clear supernatant thus obtained was used to determine the GAE activity. Protein was determined according to Bradford (1976).

The assay mixture (0.5 ml) [50 mM 2-amino-2-(hydroxymethyl) propane-1,3-diol (Tris)-HCl buffer (pH 8.5), 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 2 mM GA as substrate and enzyme extract (1.0 mg protein)] was incubated for 16 h at 30 °C in a sealed capped tube. For the determination of the GA-cleaving activity of GAE isoenzymes, native-PAGE (polyacrylamide gel electrophoresis) of the enzyme extract was run, and a gel strip was cut and stained with β-naphthylacetate. Corresponding, unstained gel strips containing GAE isozymes were homogenized in the same assay buffer and incubated with GA. G produced and left over GA after the incubation period was extracted twice with diethyl ether and subjected to GC analysis. A blank with the boiled enzyme was also run simultaneously. The appearance of the G peak indicated the presence of GAE activity.

**Native-PAGE**

Native-PAGE was performed as per Laemmli (1970). The separating (1.0 mm thick) and stacking gels (1.0 mm thick) were 13 and 5%, respectively. As electrophoresis buffer, 0.25 M Tris and 1.92 M glycine, pH 8.3, was used. An equal amount of leaf protein (1 mg) samples properly mixed with 10% sucrose and 0.1% bromophenol blue were loaded into the gel well by a micropipette. The electrophoresis was carried out at 4 °C for 3 h at 15 mA constant current. After electrophoresis, gels were stained for esterase by incubation (about 10 min in the dark at 37 °C) in a solution containing 3 mg β-naphthylacetate and 10 mg Fast blue RR salt (Sigma-Aldrich, New Delhi, India) dissolved in 50 ml 0.025 M Tris-HCl buffer, pH 7. The band volume (non-specific activity) of each isoenzyme was measured by a gel scanner (Sharp J X 330 Pharmacia Biotech, Uppsala, Sweden).

**Phosphatase activity assay**

Phosphatase activity was measured as described by Nah et al. (2001). The enzyme extract (0.1 mg) was assayed in a total volume of 3 ml containing 100 mM Tris-HCl/Tris-maleate (pH 7.5), 2 mM 2-mercaptoethanol, 10 mM MgCl₂ and 0.2 mM GPP. Lyophilized GPP was dissolved in 50 mM (NH₄)₂CO₃ (pH 9.0) (Croteau and Karp, 1979). After 4 h of incubation at 30 °C, the reaction was stopped, the mixture was vortexed, and hydrolysis products of GPP were extracted with hexane. The GPP hydrolyzing activity of phosphatases was expressed as μmol geraniol produced mg protein⁻¹.

**Results and Discussion**

**Developmental changes in monoterpene content and composition**

The accumulation of essential oil and monoterpenes (GA and G) in the mutant GRL-1 was
measured as a function of leaf development from day 10 after leaf emergence (leaf area 16.0 cm² leaf⁻¹) until senescence (50 d, leaf area 64 cm² leaf⁻¹) (Fig. 2A). In parallel, the monoterpene accumulation was also studied in the leaves at different positions (from inside to outside) within a lemongrass tiller (50-d-old) (Fig. 2B). The accumulation pattern showed that the monoterpene content of the essential oils rapidly increased from day 10 to day 40 of leaf development and then declined (Fig. 2A). The monoterpene content of the essential oils isolated from leaves of various positions of a tiller also increased in a way similar to that observed for the leaf development with relatively higher values from the 1st to the 3rd leaf (Fig. 2B). The total monoterpene content was dependent of the leaf age, young leaves having the highest levels per gram fresh weight getting progressively lower with leaf maturation and expansion. Previous studies on lemongrass and palmarosa have clearly shown that essential oils are exclusively biosynthesized in young and rapidly growing leaves (Singh et al., 1991; Sangwan et al., 1993; Dubey and Luthra, 2001; Dubey et al., 2003; Ganjewala and Luthra, 2007a, b).

Also, monoterpene biosynthesis and accumulation is not only a tissue-specific activity, but also depends on the developmental stage of the concerned organ/plant parts as well (Gershenzon et al., 1989, 2000; McConkey et al., 2000). Here, the changes observed in the monoterpene content and composition of the developing GRL-1 leaves substantiate the characteristic patterns of monoterpene accumulation in plants with a higher rate of monoterpene biosynthesis and accumulation during early growth stages, viz. 15–30 d, 1st to 3rd leaf position and in the proximal part of leaf (Figs. 2A, B). Similar developmental changes in the monoterpene content and composition in leaves and fruits of several plant species have been reported previously (Porter et al., 1983; Croteau et al., 1981; Bouwmeester et al., 1998; McConkey et al., 2000; Dubey et al., 2000, 2003; Singh et al., 1990; Sangwan et al., 1993). Therefore the results presented here are consistent and additional to those published previously.

Similarly, the monoterpene compositions also varied significantly during leaf development. The portions of GA in essential oils declined corresponding to an increase in the G level (Figs. 3A, 3B).
B, C). The level of GA rapidly turned down from 59% in 10-d-old leaves to 3% in 50-d-old leaves. A similar trend in the GA level (from 35% to ~1%) was observed from inside (1st) to outside (6th) leaf positions in 50-d-old tillers. The decrease in GA, however, was accompanied by a corresponding increase in the level of G from 33% to 91% from day 10 to day 50 of leaf development and from 45% to 95% from 1st to 6th position (Figs. 3A, B).

Similar compositional changes in the monoterpene content and composition were also recorded in the essential oil content and composition of 15-d-old leaves as they have the highest capacity to synthesize and accumulate essential oils. Changes in the essential oil content are often accompanied by alterations in the monoterpene (GA and G) content and composition as it was demonstrated in the GRL-1 mutant leaf essential oil (Figs. 3A, B, C). These compositional shifts in monoterpenes are quite common in several plants for example in *Mentha* (McConkey et al., 2000; Gershenzon et al., 2000). The monoterpene content in all these

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**Fig. 3.** Monoterpene (geranyl acetate and geraniol) composition and GAE activity in lemongrass (*Cymbopogon flexuosus*) mutant cv. GRL-1. (A) during leaf development from 10 to 50 d, (B) in leaves detached from positions 1 to 6 in 50-d-old tillers, and (C) within an immature (15-d-old) leaf from the proximal to the distal region. The experiment was repeated three times with a similar result.
taxa depended on the age of the leaf/organ with young leaves/organs having the highest levels, and then it remained relatively constant over the rest of leaf/organ life.

**GAE activity in developing lemongrass leaves**

The GAE activity significantly varied during the leaf developmental stages studied (Figs. 3A, B, C). The GAE activity was comparatively higher during the early stages, viz. in the 1st leaf and basal stages (15-d-old leaf), than in their later stages of leaf development. A similar profile of GAE activity has been observed previously in palmarosa (*Cymbopogon martinii*) by Dubey and Luthra (2001) and Dubey *et al.* (2003). These authors found that the GAE activity markedly varies as a function of inflorescence development in palmarosa (*C. martinii*). The GAE activity steadily decreases with an increase in the age of inflorescence with the highest value in the immature stage of inflorescence. Simultaneously, the level of GA also decreases with a corresponding increase in the amount of G. Hence, the GAE activity is consistent with the monoterpene composition of the essential oil in palmarosa. Our results of the GAE activity determination in the mutant cv. GRL-1 are in full agreement with the results observed in palmarosa. Thus, our results substantiate further the role of GAE in the production of G in *Cymbopogon*. The temperature and pH optima for GAE of palmarosa and the mutant cv. GRL-1 are almost similar, with temperatures of 30 °C and pH values of 8.5 (50 mM Tris-HCl). Before the report on GAE from palmarosa and lemongrass, menthyl acetate esterase (MAE) was the only esterase known from *Mentha* species correlated with the accumulation of monoterpentenyl esters (Croteau and Hooper, 1978; Wermann and Knoor, 1993). In the mutant cv. GRL-1, it is presumed that G is produced as the result of action of either recently reported geraniol synthase (GS) (Iijima *et al.*, 2004; Yang *et al.*, 2005; Michiho and Honda, 2007) or previously reported phosphatase on GPP (Perez *et al.*, 1980; Nah *et al.*, 2001). Although, G gets acetylated to GA by transacylase very shortly after its formation from GPP, GA thus produced simultaneously gets hydrolyzed by highly active co-occurring GAE producing G in the immature leaf developmental stage. Hence, the transacylase activities could not be detected in the crude enzymatic preparation from mutant GRL-1 leaves probably due to the co-occurrence of very high GAE activity. In contrast to palmarosa and lemongrass, a transacetylase, acetyl-CoA: geraniol acetyltransferase, has been cloned and characterized from rose petals, which catalyzes the formation of GA from G and is developmentally regulated (Shalit *et al.*, 2003).

Phosphatases extracted from 15-d-old leaves and incubated with GPP for 4 h in Tris-maleate (pH 7.5) and Tris-HCl (pH 7.5) buffers produced 58% and 48% G, respectively. This study is based on the fact that phosphatases from flavedo of citrus are able to hydrolyze prenyl pyrophosphates (GPP, FPP and NPP) to their corresponding alcohols (Perez *et al.*, 1980). Here we assigned phosphatase as GPPase that removes the PPI from GPP and produces G in a way similar to the phosphatases of citrus that hydrolyze the prenyl diphosphates breaking the phosphoester bond (Perez *et al.*, 1980). However, the structure of G, whose carbon skeleton is identical to that of its precursor geranyl diphosphate, supports an alternative mechanism of simply breaking the phosphoester bond by phosphatases. Although, our results favour the phosphatase-mediated formation of G, here at a time whether G is synthesized by the action of phosphatases or recently reported monoterpene synthases are not very clear in the lemongrass mutant cv. GRL-1.
GAE isozymes in lemongrass

Fig. 4 depicts the major GAE isoenzymes in polyacrylamide gel visible after staining with the artificial substrate \( \beta \)-naphthylacetate. Their GA cleaving activities markedly varied during leaf development (see Tables I–III). The GA cleaving activity of GAE isozymes was measured in the 1st, 3rd and 5th leaf of the lemongrass tiller. The GA cleaving activity was recorded maximum for GAE-I in the 1st leaf position which then significantly declined in the 3rd leaf position and remained more or less similar thereafter. Unlike GAE-I, the GA cleaving activity of GAE-III was recorded lowest in the 1st leaf position which increased rapidly in the 3rd leaf position and came down to the minimum level in the 5th leaf position. However, no definite trend in the GA cleaving activity was observed for the GAE-III, rather it frequently varied from the 1st to 6th leaf position with a sharp peak in the 3rd leaf. Surprisingly, the GA cleaving activity of GAE-II isoenzyme remained almost similar from the 1st to 6th leaf position in the tiller. Identical GA cleaving activity profiles of these three isozymes were observed in three parts, basal, middle and apical, of 15-d-old leaves. The results clearly suggested that varying expressions of GAE isoenzymes are in accordance with the monoterpenes compositions during GRL-1 leaf development. For quantitative fluctuation in the GAE isoforms, it has been suggested that the genes encoding different isoenzymes may express at different times during leaf development as documented for *Linum usitatissimum* (Yurenkova *et al.*, 1995). Obviously, GAE-I among others is most prominent in determining the monoterpenes compositions of the essential oils. The polymorphism and abundance of the GAE enzyme suggest that it plays important roles in plant growth and differentiation (Yurenkova *et al.*, 1995). In our previous study we employed esterase polymorphism where GAE-I has served as a biochemical marker for the identification of lemongrass species and cultivars (Ganjewala and Luthra, 2007c). Lemon-grass species and cultivars could be differentiated

<table>
<thead>
<tr>
<th>Leaf age [d]</th>
<th>GAE-I</th>
<th>GAE-II</th>
<th>GAE-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>55.0</td>
<td>15.0</td>
<td>11.0</td>
</tr>
<tr>
<td>15</td>
<td>31.0</td>
<td>17.0</td>
<td>13.0</td>
</tr>
<tr>
<td>20</td>
<td>28.0</td>
<td>15.0</td>
<td>9.0</td>
</tr>
<tr>
<td>30</td>
<td>23.0</td>
<td>16.0</td>
<td>12.0</td>
</tr>
<tr>
<td>40</td>
<td>14.0</td>
<td>17.0</td>
<td>24.0</td>
</tr>
<tr>
<td>50</td>
<td>3.0</td>
<td>5.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Table II. GA cleaving activity of GAE isoenzymes from 1st to 6th leaf in a 50-d-old tiller of lemongrass mutant cv. GRL-1.

<table>
<thead>
<tr>
<th>Leaf position</th>
<th>GA cleaving activity (% GA hydrolyzed/16 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAE-I</td>
<td>GAE-II</td>
</tr>
<tr>
<td>1</td>
<td>76.0</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>48.0</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>45.0</td>
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<tr>
<td>6</td>
<td>–</td>
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</tbody>
</table>

Table III. GA cleaving activity of GAE isoenzymes within 15-d-old leaves from the proximal to the distal region in lemongrass mutant cv. GRL-1.

<table>
<thead>
<tr>
<th>Leaf part</th>
<th>GA cleaving activity (% GA hydrolyzed/16 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAE-I</td>
<td>GAE-II</td>
</tr>
<tr>
<td>Basal</td>
<td>48.0</td>
</tr>
<tr>
<td>Middle</td>
<td>25.0</td>
</tr>
<tr>
<td>Apical</td>
<td>18.0</td>
</tr>
</tbody>
</table>

The experiment was repeated three times and the average value has been given here.
as citral- or geraniol-rich based on the presence or absence of GAE-I. However, further studies are needed to resolve the GAE polymorphism and to find its implications in improving the quality of essential oils.

The effects of phenylmethylsulfonyl fluoride (PMSF), p-chloromercuribenzoic acid (pCMB), and metal ions (MgCl₂ and ZnCl₂) were tested on GAE isoenzymes (Fig. 5). pCMB totally inactivated the GAE-III but did not affect the GAE-I and GAE-II. PMSF and the metal ions Mg²⁺ and Zn²⁺ in their used concentrations were not inhibitory to any of the GAE isozymes. The enzyme inhibitor pCMB totally inactivated the GAE-III isozyme suggesting it either as acetylene or cholinesterase which is inhibited by the sulphydryl reagent pCMB (Muralidharan et al., 1996). Lack of inhibition of the GAE isozyme activity by PMSF suggests that these esterases lack a serine residue at the active site.

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