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An esterase is involved in geraniol production during palmarosa inflorescence development

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Abstract

Total incorporation of exogenously administered [2-^{14}C]acetate into essential oil of palmarosa (*Cymbopogon martinii*) was found to be relatively higher than that of either [U-^{14}C]sucrose or [U-^{14}C]glucose during inflorescence development. Among the major essential oil constituents, biogenesis of geranyl acetate was much higher than that of geraniol. Alkaline hydrolysis of [^{14}C]labeled geranyl acetate revealed that the majority of the label incorporated into geranyl acetate was present in the geraniol moiety, indicating that only newly synthesized geraniol gets acetylated to form geranyl acetate. Geranyl acetate cleaving esterase (GAE) activity followed a similar pattern during both in vivo and in vitro inflorescence development, with maximum activity at immature inflorescence stages, suggesting the involvement of GAE in geraniol production during inflorescence development. Five esterase isozymes (Est-A to E) were detected in the enzymic fraction of palmarosa inflorescence and all showed GAE activity, with Est-B being significantly increased during inflorescence development. The role of GAE in geraniol production and improving the palmarosa oil quality is discussed.

Keywords: *Cymbopogon martinii*; Gramineae; Essential oil; Geraniol; Geranyl acetate esterase; Monoterpenoids; Palmarosa inflorescence

1. Introduction

Monoterpenoids are best known as constituents of the essential oils and defensive oleoresins of various aromatic plant species (Singh et al., 1989b; Mahmoud and Croteau, 2002). In higher plants, monoterpenes participate in a wide variety of functions and are synthesized and stored in various types of distinct and highly specialized structures such as resin ducts, secretory cavities, latex vessels and epidermal glands found in several aromatic plants (McGarvey and Croteau, 1995; Wise and Croteau, 1999). Several biochemical and histochemical studies have revealed that these specialized structures are the actual site of monoterpene biosynthesis that appear only in particular organs at various stages of plant development (McCaskill et al., 1992; Wise and Croteau, 1999).

The essential oils of palmarosa (*Cymbopogon martinii*) inflorescences (flowering tops) mainly contain two acyclic monoterpenoids, geraniol and geranyl acetate. While palmarosa oil is used in a wide range of perfumes, soaps, cosmetics, toiletry, and tobacco products and also has some pharmaceutical properties as well (Dubey et al., 2000), its biosynthesis is not well established, since the route to isopentenyl diphosphate (IPP) and to geraniol (geranyl acetate) biosynthesis in palmarosa has not yet been clarified. On the other hand, various biochemical and physiological studies have been carried out in lemongrass (*Cymbopogon flexuosus*) (Singh and Luthra, 1988; Singh et al., 1989a, 1990, 1991; Sangwan et al., 1993a,b).

Geraniol is known to be derived from geranyl diphosphate (GPP), via head to tail condensation of IPP with dimethylallyl diphosphate (DMAPP). IPP is in turn synthesized from cytoplasmic acetate-mevalonate or recently discovered plastidic non-mevalonate (pyruvate/triose-

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phosphate) pathway (Luthra et al., 1999; Rohmer, 1999; Mahmoud and Croteau, 2002). In some plant species geraniol is known to be synthesized via the non-mevalonate pathway (Eisenreich et al., 1997; Luan and Wüst, 2002).

Monoterpenoids are mainly derived through interconversions, such as stereospecific isomerizations, acetylations, deacetylations, cyclizations and dehydrogenations (McGarvey and Croteau, 1995). These metabolic transformations are very important determinants of the oil composition. Although much work has been done on enzymatic transformations of cyclic monoterpenoids (Wise and Croteau, 1999), information on interconversions of acyclic monoterpenes is very limited. Most secondary modifications and transformations take place after the synthesis of the acyclic/cyclic monoterpenoids and/or their derivatives that naturally exist in plants. One such important interconversion was observed during palmarosa inflorescence development, where the proportion of geranyl acetate in essential oil was significantly decreased with a corresponding increase in geraniol (Dubey et al., 2000). The level of geraniol in palmarosa oil presumably depends on the relative activities of three metabolic steps: (i) removal of diphosphate from GPP, (ii) acetylation of geraniol to geranyl acetate and (iii) deacetylation of geranyl acetate to form geraniol. Our earlier studies with palmarosa inflorescence suggested that deacetylation of geranyl acetate to geraniol is more dominant during inflorescence development (Dubey et al., 2000), and more recently we have detected a geranyl acetate cleaving esterase (GAE) activity in palmarosa inflorescence (Dubey and Luthra, 2001). Despite the great commercial importance and demand for geraniol isolated from palmarosa, the biosynthesis of geraniol and enzymes involved in the process have not been elucidated. As the geraniol percentage is an index of palmarosa oil quality, the enzymology of this interconversion is of great interest. In this paper we report for the first time the biosynthesis of essential oil and its major monoterpene constituents using [^{14}C]labeled precursors and the important role of an esterase in geraniol production during palmarosa inflorescence development.

2. Results and discussion

2.1. Essential oil biosynthesis in palmarosa inflorescences using [^{14}C]radiolabeled precursors

Essential oil biosynthesis during palmarosa inflorescence development was monitored through the total incorporation of [^{14}C]labeled precursors into essential oil. At all inflorescence stages, the total incorporation of [2-^{14}C]acetate into oil was relatively higher than that of [U-^{14}C]sucrose and [U-^{14}C]glucose (Table 1). Irrespective of the ^{14}C-radiolabeled precursors, the [^{14}C]radioactivity incorporated into essential oil was substantially higher at inflorescence with partially opened spikelets (stage II) and then decreased, except in case of [U-^{14}C]glucose, where [^{14}C]radioactivity incorporation into oil was slightly higher at inflorescence with unopened spikelets (stage I) compared to stage II (Table 1). With all three radiolabeled precursors, the data expressed as pmol/g fresh weight of spikelets also showed higher [^{14}C]radioactivity incorporation into oil at inflorescence stage II and decreased thereafter (data not shown). Since sucrose provides four acetate equivalents and glucose two, on that basis the total incorporation of [^{14}C]acetate at stages I and II was roughly equivalent to the other two [^{14}C]radiolabeled precursors (except in case of [U-^{14}C]glucose at stage II); however, during the later stages of inflorescence growth, total incorporation of [^{14}C]acetate was relatively higher than that of either [U-^{14}C]sucrose or [U-^{14}C]glucose. Although substantial essential oil biosynthetic capacity was retained until inflorescence with partially mature spikelets (stage IV), the previously observed decrease in essential oil content just after the inflorescence stage I (Dubey et al., 2000) suggests that the catabolic processes become predominant as the inflorescence matured. It seems more likely that at inflorescence stage I, the net synthesis greatly exceeds turnover and, therefore, terpenes accumulate. Earlier studies involving the leaves of lemongrass and citronella (two *Cymbopogon* species) suggested that [2-^{14}C]acetate rather than [U-^{14}C]sucrose was preferentially incorporated into monoterpenes

Table 1
Incorporation of [^{14}C]radiolabeled precursors into essential oil at various stages of palmarosa inflorescence development. LSD values at $P \leqslant 0.01$ for [^{14}C]radioactivity incorporated into essential oil using acetate, sucrose and glucose as precursors were 9.86, 8.60 and 7.12, respectively

Inflorescence stage	Radioactivity incorporated (pmol/100 spikelets)		
	[2-^{14}C]acetate[a]	[U-^{14}C]sucrose[a]	[U-^{14}C]glucose[a]
I. Unopened spikelets	206.40±9.6	43.00±2.8	92.84±7.6
II. Partially opened spikelets	508.39±20.5	138.84±6.6	73.00±4.8
III. Fully opened spikelets	394.27±16.8	32.02±2.0	43.23±2.6
IV. Partially mature spikelets	239.14±11.4	16.03±1.2	24.56±1.8
V. Fully mature spikelets	49.04±3.0	7.96±0.7	6.17±0.5

[a] In each case, 5 μCi of [^{14}C]radiolabeled precursor was administered. The concentration of each radiolabeled substrate was subsequently adjusted to 1 μmol by adding cold (unlabeled) substrate.

(Singh et al., 1990; Luthra et al., 1993). Incorporation studies with secretory cell clusters from peppermint (*Mentha piperita*) leaves indicated that the incorporation of [^{14}C]acetate into monoterpenes was roughly equivalent to that of [^{14}C]sucrose (McCaskill et al., 1992).

2.2. Incorporation of [2-^{14}C]acetate into essential oil and its major constituents

The relative distribution of the radioactivity into the major oil constituents, geraniol and geranyl acetate, revealed that the [2-^{14}C]acetate incorporated into geranyl acetate was much higher compared to that in geraniol (Fig. 1). Similar to essential oil biosynthesis, the capacity of the inflorescence to synthesize geranyl acetate decreased substantially after the inflorescence stage II (Fig. 2), and the biogenesis of geranyl acetate was generally higher than that of geraniol (as determined by their relative% in the oil radioactivity) at all the developmental stages except at inflorescence stage V (Fig. 1). At inflorescence stage I about 90% of the radioactivity incorporated into essential oil was recovered in geranyl acetate. As the spikelets opened, the relative percentage of the radioactivity in geranyl acetate decreased; and this is accompanied by a small increase in the radioactivity in geraniol. In mature inflorescence a major shift in tracer incorporation into unidentified components was also observed (data not shown). Although geranyl acetate content was relatively less compared to geraniol at all stages of inflorescence development (Dubey et al., 2000), the biogenesis of geranyl acetate was found to be significantly much higher than that of geraniol, which might be due to the acetylation of a preexisting endogenous pool of free geraniol with [^{14}C]labeled acetyl-CoA. However, when [^{14}C]geranyl acetate from the oil was subjected to alkaline hydrolysis with 10% ethanolic KOH, the majority of [^{14}C]radioactivity (60–90%) was in the geraniol moiety, with the acetate moiety contributing just a small portion of the radioactivity present in the geranyl acetate (Fig. 2). Thus, only newly synthesized geraniol acetylates to form geranyl acetate.

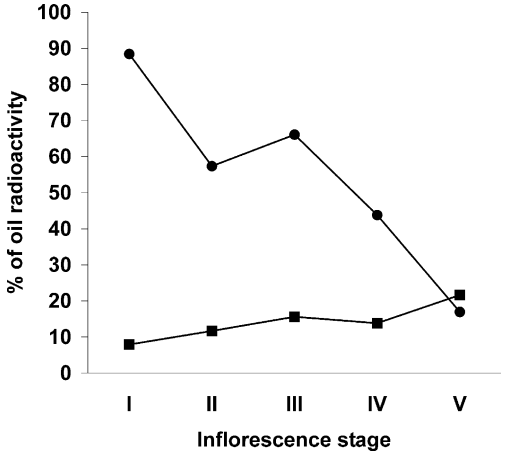

Fig. 1. Relative % distribution of essential oil [^{14}C]radioactivity into geranyl acetate (filled circles) and geraniol (filled squares) using radiolabeled acetate as precursor during palmarosa inflorescence development.

The relative distribution of [^{14}C]radioactivity into free- and acetylated geraniol (the geraniol moiety of the geranyl acetate) during inflorescence development indicates that incorporation into acetylated geraniol was much higher than that in free geraniol, except at inflorescence stage V (Fig. 3). The biosynthetic capacity of the inflorescence to synthesize total geraniol, expressed as the sum of [^{14}C]radioactivity incorporated into free geraniol and acetylated geraniol, increased from inflorescence stage I to stage II, and decreased substantially therafter until stage V (Fig. 3). At inflorescence stage I, about 90% of the total geraniol synthesized has undergone acetylation to form geranyl acetate, and the total percentage of synthesized geraniol, decreases thereafter (Fig. 3). The biosynthetic capacity of tissue to synthesize geranyl acetate decreases with a substantial increase in geraniol biosynthesis during the inflorescence development. This result is consistent with earlier observation in which geranyl acetate proportion was decreased with a concomitant increase in the geraniol proportion in the essential oil (Dubey et al., 2000).

2.3. In vivo hydrolysis of labeled geranyl acetate synthesized from [2-^{14}C]acetate during in vitro culture

To study the in vivo hydrolysis of [^{14}C]geranyl acetate, palmarosa stage I inflorescences (administered with

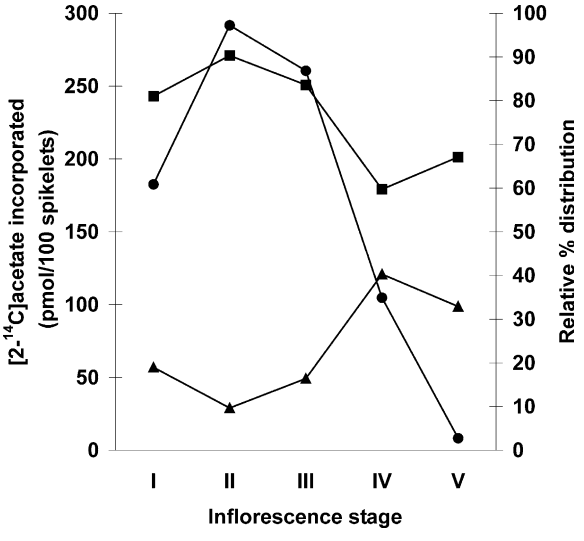

Fig. 2. Incorporation of [2-^{14}C]acetate into geranyl acetate (filled circles), and relative % distribution of radioactivity incorporated into geraniol (filled squares) and acetate (filled triangles) moiety of geranyl acetate during palmarosa inflorescence development. The pmol incorporated into geranyl acetate has been calculated from [^{14}C]radioactivity incorporated into essential oil and the relative % distribution of oil radioactivity into geranyl acetate.

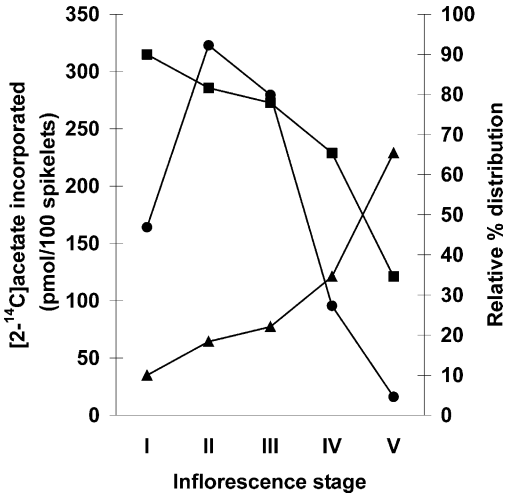

Fig. 3. Incorporation of [2-^{14}C]acetate into total geraniol (filled circles), and % distribution of radioactivity incorporated into acetylated geraniol (filled squares) and free geraniol (filled triangles) during palmarosa inflorescence development. The pmol incorporated into the total geraniol represents the sum total of the ^{14}C label incorporated into free- and acetylated geraniol.

[2-^{14}C]acetate) were cultured for 10 days in Hoagland solution containing 2% sucrose. Relative distribution of the [^{14}C]radioactivity into geranyl acetate and geraniol during the culture period showed substantial loss in [^{14}C]radioactivity from geranyl acetate with a corresponding increase in that of geraniol, and the radioactivity in unknown constituents remained more or less constant throughout the inflorescence development (Fig. 4A).

Ontogenic changes and the accumulation of essential oil and its major constituents, geraniol and geranyl acetate, were also studied during in vitro development of palmarosa inflorescence for the same culture period. The period from the opening of the spikelets to complete maturity in the intact plant under field conditions was approximately one month, while during in vitro culture the process of spikelets development was considerably accelerated. Although fresh weight (FW) and dry weight (DW) of the spikelets were relatively lower, the pattern of FW and DW accumulation during in vitro culture was very similar (data not shown) to that observed under field conditions (Dubey et al., 2000). The essential oil content was found to be more or less constant until the sixth day of the culture period; it then slightly increased through the 8th day of culture period and decreased thereafter (data is presented later in Fig. 6). The relative proportion of geraniol and geranyl acetate in the essential oil indicated a constant decrease in the geranyl acetate percentage, with corresponding increase in the geraniol percentage during inflorescence development (Fig. 4B). The results also showed that the geranyl acetate proportion in the oil was relatively less as compared to geraniol at all stages of inflorescence development during the culture period; however, beginning at the second day of the culture period, the biogenesis of geranyl acetate was much higher than that of geraniol (Fig. 4A and B). The changes in essential oil content and its major constituents during in vitro studies with developing palmarosa inflorescence were very similar to that observed in earlier studies with an intact plant system (Dubey et al., 2000). In some plant species it has been reported that detachment of the plant parts may cause artefacts in the results related to radiotracer studies (Gershenzon et al., 1993). However, our studies with palmarosa inflorescence show similar patterns both in the detached plant part, as well as in the intact plant

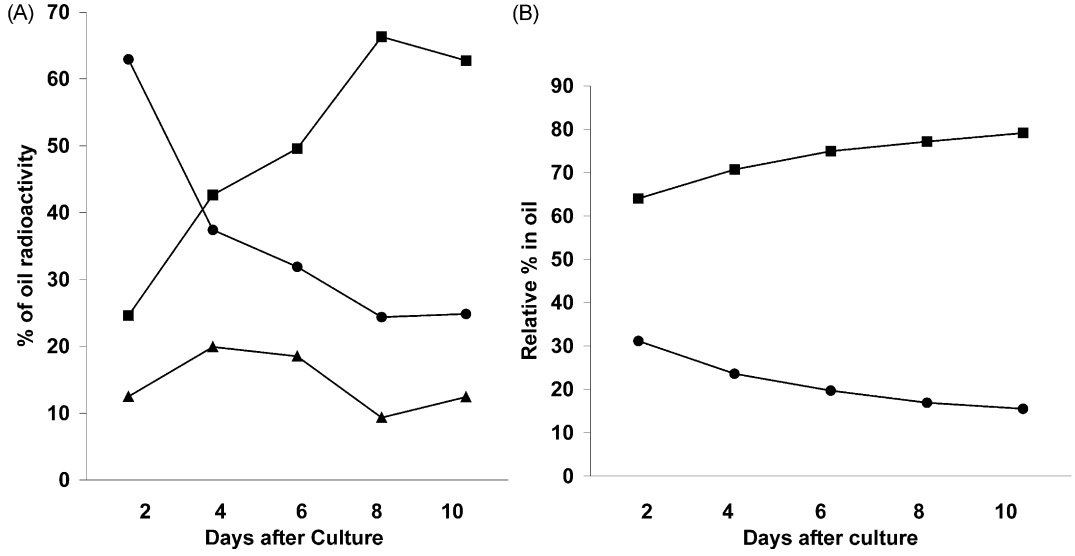

Fig. 4. (A) Relative % distribution of [^{14}C]radioactivity into geranyl acetate (filled circles), geraniol (filled squares) and unknown constituents (filled triangles), and (B) relative proportion of geraniol (filled squares) and geranyl acetate (filled circles) in essential oil, during palmarosa inflorescence development under in vitro conditions. The experiment was repeated three times with the results following a similar pattern. Representative data from a single experiment has been given.

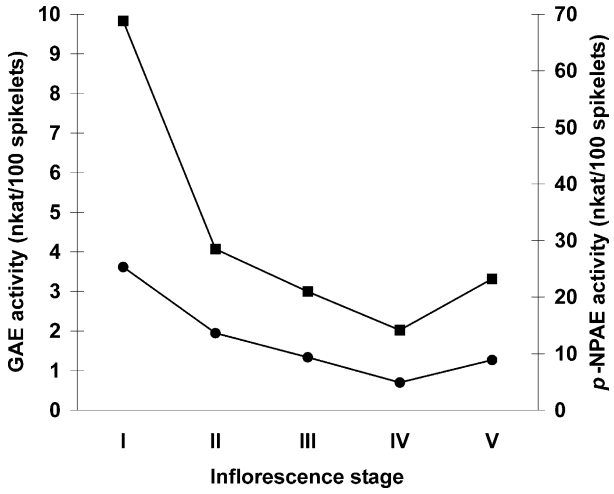

Fig. 5. Activity profiles of geranyl acetate-cleaving esterase (GAE) (filled circles) and *p*-nitrophenyl acetate esterase (p-NPAE) (filled squares) during palmarosa inflorescence development. LSD values at $P \leqslant 0.01$ were 0.06 and 0.30, respectively.

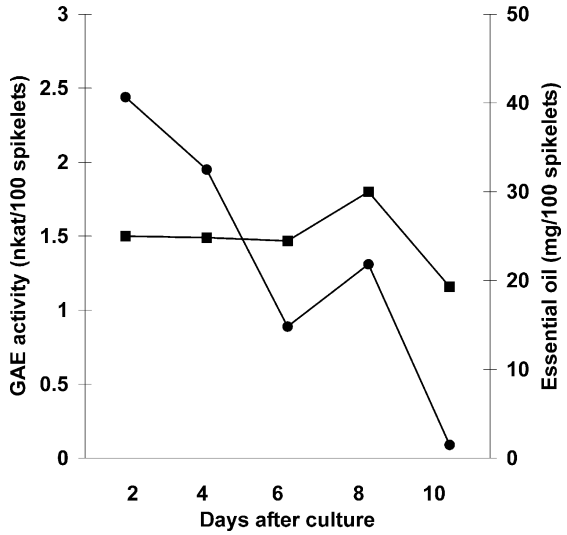

Fig. 6. Activity of geranyl acetate-cleaving esterase (GAE) (filled circles) and essential oil content (filled squares) during in vitro culture of palmarosa inflorescence development. The experiment was repeated three times, and representative data from a single experiment has been given.

system in relation to accumulation and biosynthesis of essential oil and its major constituents, geraniol and geranyl acetate.

2.4. GAE activity during in vivo and in vitro palmarosa inflorescence development

Both in vivo and in vitro biosynthetic studies have suggested the predominant role of an esterase in producing geraniol by the hydrolysis of geranyl acetate during palmarosa inflorescence development. Both GAE and *p*-nitrophenyl acetate catalysing esterase (*p*-NPAE) activities were detected during various inflorescence developmental stages. The GAE activity was maximum at inflorescence stage I and thereafter decreased significantly until inflorescence stage IV, followed by a sudden increase at inflorescence stage V (Fig. 5). At inflorescence stage I, GAE activity was almost 3 times that of inflorescence stage V; a similar trend was also observed for non-specific esterase (*p*-NPAE) activity (Fig. 5).

GAE activity was also studied during in vitro development of palmarosa inflorescence for 10 days of culture period. The GAE activity was maximum at the beginning of culture period (second day after culture) and then decreased significantly until the sixth day. A sudden increase in GAE activity was observed at day eight, that almost disappeared thereafter (Fig. 6). In accordance with this the essential oil content of the spikelets from in vitro developing palmarosa inflorescence also increased during the later stages (6–8 days) of the culture period and then decreased (Fig. 6). The GAE activities expressed as nkat mg protein^{-1} or gFW^{-1} of spikelets followed a similar trend in both in vivo and in vitro experiments (data not shown). Both in vivo and in vitro approaches have clearly demonstrated the potential role of GAE in producing geraniol by the hydrolysis of geranyl acetate during palmarosa inflorescence development. However, at this time we have no explanation as to why GAE activity suddenly increased during the later stages of development when inflorescence matured.

2.5. Identification of GAE activity of non-specific esterase isozymes

Five esterase isozymes were detected in palmarosa at various stages of inflorescence development, which were either slow moving (Est-A and Est-B) or fast moving (Est-C, Est-D and Est-E) on the bais of their mobility on gel (Fig. 7). The non-specific esterase activity (expressed as band intensity) of Est-B continuously increased as the inflorescence matured with maximal value at inflorescence stage V, while that of other esterase isozymes did not significantly change (data not

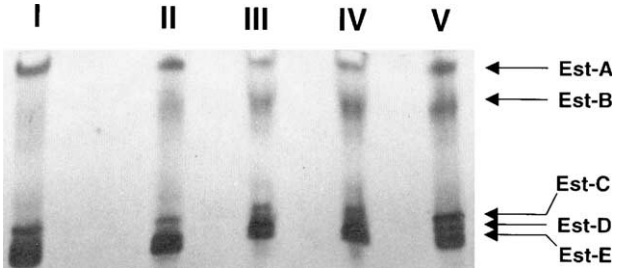

Fig. 7. Pattern of esterase isozymes during palmarosa inflorescence development. The numbers I to V represent the developmental stages. Each esterase isozyme (Est-A to E) is shown by an arrow. In each case, 100 μg protein was loaded on gel.

shown). GAE activity of all five esterase isozymes at inflorescence stage I was determined using geranyl acetate as substrate. Since the expression of Est-B significantly increased during the development (Fig. 7), we also detected the GAE activity of Est-B at inflorescence stages III and V. All five esterases (as stained by β-naphthyl acetate) showed GAE activity (Table 2). At inflorescence stage I, Est-E showed the maximum GAE activity, whereas Est-B, which was hardly seen on the gel, contained the minimum GAE activity. However, GAE activity of Est-B increased 4.4 fold from inflorescence stage I to inflorescence stage V (Table 2). This is consistent with the increased expression of this particular isozyme (Est-B) during inflorescence development.

Esterase isozymes are ubiquitous in the plant kingdom and have several known physiological functions in plant secondary metabolism. The expression of the individual isozyme depends upon the developmental stages of the plant tissue used (Dubey et al., 2001). During palmarosa inflorescence development, the significant increase in the expression of Est-B suggests an important role of this particular isozyme in the production of geraniol and perhaps involvement in some other unknown physiological process. Furthermore, it may be presumed that the sudden increase in GAE activity at a matured inflorescence stage was probably due to Est-B. The GAE activity of all five esterase isozymes suggests that the geranyl acetate-cleaving activity is one of the catalytic activities of the non-specific esterases. The enzymic fraction from palmarosa inflorescence containing GAE activity was also capable of hydrolysing some other acyclic monoterpenyl esters, such as geranyl formate and citronellyl acetate (Dubey et al., 2002). The non-pecificity of other plant esterases involved in the hydrolysis of menthyl acetate (Croteau and Hooper, 1978), pyrethroid insecticide cyfluthrin (Preiss et al., 1988), cardenolides (Kandzia et al., 1998) and methyl jasmonate (Stuhlfelder et al., 2002) have also been reported. The present study suggests that at least one esterase is involved in the production of geraniol (a commercially valuable monoterpene) during inflorescence development, which improves the oil quality of palmarosa.

Table 2
GAE activity of non-specific (β-napthyl acetate hydrolysing) esterase isozymes from native-PAGE analysis at various stages of palmarosa inflorescence development. At each inflorescence stage, the same amount of protein (100 μg) was loaded on the gel

Esterase isozymes	GAE activity (% geranyl acetate hydrolysed/ 16 h)		
	Stage I	Stage III	Stage V
Est-A	23.2	–	–
Est-B	16.2	19.2	71.1
Est-C	31.8	–	–
Est-D	31.6	–	–
Est-E	40.3	–	–

Various inflorescence stages are described in experimental section.
–, Activity was not determined.

3. Experimental

3.1. Plant material and chemicals

Palmarosa (*Cymbopogon martinii*, Roxb. Wats. Var. *motia*) plants were raised from seedlings at the experimental farm of the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. The inflorescence (flowering tops) were tagged at the time of emergence and harvested at five different developmental stages I to V: unopened spikelets, partially opened spikelets (anthers partially visible), fully opened spikelets (yellow anthers fully visible), partially mature spikelets (anthers brown and yellow) and fully mature spikelets (brown inflorescence), respectively. All results presented are based on 100 spikelets, unless otherwise specified.

Geraniol and geranyl acetate were purchased from Sarsynthase (France) and all other needed biochemicals were from Sigma Chemical Co. (USA). Solvents and other chemicals used were of high purity (analytical reagent grade). The radiochemicals [2-^{14}C] sodium acetate (0.1 mCi, 34.51 Ci/mol), [U-^{14}C] glucose (0.1 mCi, 140 Ci/mol) and [U-^{14}C] sucrose (0.1 mCi, 300 Ci/mol) were procured from Bhabha Atomic Research Centre (BARC), Trombay, India.

3.2. In vivo [^{14}C]radio-tracer studies

For in vivo incorporation studies, palmarosa inflorescences at each developmental stage were cut under water and placed in vials with the cut ends dipped in an aqueous solution (1 ml) of 5 μCi [^{14}C]radiolabeled substrate (acetate/glucose/sucrose). The final amount of each substrate was adjusted to 1 μmol by adding cold (unlabeled) substrate. The tubes were then kept in bright sunlight with successive additions of half strength Hoagland solution (Hoagland and Arnon, 1938) to keep inflorescences immersed. After 24 h of incubation the spikelets were subjected to microscale steam distillation for essential oil extraction by using a mini-Clevenger apparatus (Dubey et al., 2000). The volatile essential oil was recovered by diethyl ether extraction, and the total [^{14}C]radioactivity in the essential oil was determined using a β-liquid scintillation counter (LKB Wallace 1409, Pharmacia Biotech). The major essential oil constituents, geraniol and geranyl acetate, were separated by prep TLC on silica gel-G plates (20×20 cm, 0.5 mm) using toluene:EtOAc (96:4, v/v) as solvent system. The concentrated essential oil samples were applied along with the authentic samples of geraniol and geranyl acetate. The plates were dried at room temperature and

visualized with iodine vapours. The geraniol and geranyl acetate spots were scraped off directly into the scintillation vials or eluted with diethyl ether and used for determination of [^{14}C]radioactivity. Purity of the geraniol and geranyl acetate separated through TLC was checked by GLC as previously described (Dubey et al., 2000). The quantitative values of the various oil constituents were computed from the relative percentage data and the amount of oil from 100 spikelets.

For alkaline hydrolysis, the [^{14}C]geranyl acetate previously purified from TLC was placed in a round bottom flask containing 5 ml of 10% ethanolic KOH and fitted with a guard tube filled with fused calcium chloride. The reaction mixture was incubated at room temperature with constant stirring for 24 h. After incubation, distilled water (1 ml) was added and the reaction mixture was extracted with diethyl ether (3×5 ml) to recover the geraniol. 1 N HCl were added to the aqueous phase to neutralize KOH. Aliquots of the diethyl ether and aqueous phases, were each placed in a scintillation vial for [^{14}C]radioactivity counting. [^{14}C]radioactivity incorporated (pmoles) into essential oil was calculated, as described by Singh et al. (1991).

3.3. In vitro culture studies with palmarosa inflorescence

Palmarosa inflorescence with partially opened spikelets (stage II) were administered [2-^{14}C]acetate (5 μCi) by dipping cut ends in the radiolabeled solution in test tubes. After complete uptake, the inflorescence was transferred in half strength Hoagland solution containing 2% sucrose and 0.05% sporidex (a broad range antibiotic), which was changed every 3 days and cultured for 10 days under in vitro glasshouse conditions. The spikelets were harvested after every two days for essential oil extraction and GLC analysis (Dubey et al., 2000). The essential oil was subjected to TLC separation of geraniol and geranyl acetate. [^{14}C]radioactivity, incorporated in total essential oil and its major constituents, geraniol and geranyl acetate, was also determined. A second set of palmarosa inflorescence of the same stage was also cultured in a similar manner without feeding radiolabeled precursor to study the accumulation pattern of essential oil and its major oil constituents.

3.4. Enzyme extraction and assay

Enzyme extraction and GAE activity assay were exactly as previously described (Dubey and Luthra, 2001). In brief, samples (inflorescences) were collected at various developmental stages from palmarosa inflorescence, and the tissues (spikelets) were homogenized in 0.1 M NaPi buffer (pH 6.5) containing 50 mM sodium metabisulphite, 10 mM β-mercaptoethanol, 10 mM ascrobic acid, 0.25 M sucrose and 1 mM EDTA-Na_2, in the presence of 50% (w/w) insoluble PVPP (polyvinyl polypyrrolidone). The slurry was squeezed through four layers of muslin cloth and centrifuged at 15,000 g for 60 min. Purified amberlite XAD-4 resin (50% of the tissue weight) was added to the supernatant and kept for 5 min at 4 °C to remove endogenous terpenes. The clear filtrate from the slurry was used to determine the GAE and non-specific esterase activities using geranyl acetate and *p*-nitrophenyl acetate as substrates, respectively, and for isozymic analysis of esterases.

GAE activity was determined by GLC monitoring of geraniol produced by hydrolysis of geranyl acetate as previously described (Dubey and Luthra, 2001). The assay mixture, containing 0.05 M Tris-HCl buffer (pH 8.5), 5 mM $MgCl_2$, 1 mM DTE, 2 mM geranyl acetate in a total volume of 0.5 ml, was incubated with enzyme extract at 30 °C for 3 h, and after incubation, the mixture was extracted with diethyl ether for GLC analysis. One unit of GAE activity (nkat) was defined as the amount of enzyme required to hydrolyse one nmole of geranyl acetate per second. Non-specific esterase activity was measured by monitoring the production of *p*-nitrophenol by the enzymatic hydrolysis of *p*-nitrophenyl acetate. The assay system consisted of 0.1 M NaPi buffer (pH 7.0), *p*-nitrophenyl acetate (1.2 μmol, dissolved in acetone) and the enzyme extract in a total volume of 3 ml. The reaction was initiated with the addition of *p*-nitrophenyl acetate. The increase in absorbance due to release of *p*-nitrophenol was measured at 400 nm for 5 min and quantified by using *p*-nitrophenol standard curve (0.02–0.2 μmol). One unit (nkat) of the enzyme activity was defined as the amount of enzyme required to produce one nmole of *p*-nitrophenol per second. Total soluble protein was estimated by Bradford's method (Bradford, 1976) using bovine serum albumin as standard.

3.5. Isozymic analysis of esterases and detection of their GAE activity

Polyacrylamide gel electrophoresis under non-denaturing conditions (native-PAGE) for esterase isozymes was carried out according to Laemmli's (1970) procedure. After complete run, the gels were stained for non-specific esterases by incubating in the 0.025 M Tris-HCl buffer (pH 7.0) containing β-naphthyl acetate (3 mg/10 ml) and Fast blue RR salt (1 mg/ml) at 37 °C for 10–15 min in the dark. After incubation, the gels were washed with distilled water and then photographed. The bands that appeared indicated the presence of non-specific esterase activity. The band on gel was scanned by gel scanner (sharp JX 330, Pharmacia Biotech) to determine the activity (as determined by band intensity) of each esterase isozyme. A single strip containing one well was cut from the gel and stained for non-specific esterases as described above. The gel strips were excised

corresponding to each band (esterase isozyme) for detection of GAE activity. These gel strips were homogenized in the assay buffer along with substrate geranyl acetate (as described in the previous section) and incubated at 30 °C in a capped tube for 16 h. A control was also used without enzyme extract to check any non-enzymic hydrolysis of the substrate during the incubation period. After incubation, the reaction mixture was extracted with ethyl ether, and the product geraniol was analysed by GLC (Dubey and Luthra, 2001).

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References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitative determination of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Croteau, R., Hooper, C.L., 1978. Metabolism of monoterpenes: Acetylation of (-)-menthol by a soluble enzyme preparation from peppermint (*Mentha piperita*) leaves. Plant Physiol 61, 737–742.

Dubey, V.S., Mallavarapu, G.R., Luthra, R., 2000. Changes in the essential oil content and its composition during palmarosa (*Cymbopogon martinii* (Roxb.) Wats. Var. *motia*) inflorescence development. Flav. Frag. J. 15, 309–314.

Dubey, V.S., Luthra, R., 2001. Biotransformation of geranyl acetate to geraniol during palmarosa (*Cymbopogon martini*, (Roxb.) Wats var. *motia*) inflorescence development. Phytochemistry 57, 675–680.

Dubey, V.S., Luthra, R., Kumar, S., 2001. Plant esterases: physiological functions and their role in secondary metabolism. J. Med. Arom. Plant Sci. 22, 273–287.

Dubey, V.S., Luthra, R., Naqvi, A.A., Kumar, S., 2002. Stable esterase obtained from palmarosa. US Patent No. 6,387,678.

Eisenreich, W., Sagner, S., Zenk, M.H., Bacher, A., 1997. Monoterpenoid essential oils are not of mevalonoid origin. Tetrahedron Lett. 38, 3889–3892.

Gershenzon, J., Murtagh, G.J., Croteau, R., 1993. Absence of rapid terpene turnover in several diverse species of terpene-accumulating plants. Oecolog 96, 583–592.

Hoagland, D.R., Arnon, D.I., 1938. The water culture method for growing plants without soil. Circ. Calif. Agric. Exp. Stat 347, 32.

Kandzia, R., Grimm, R., Eckerskorn, C., Lindermann, P., Luckner, M., 1998. Purification and characterization of lanatoside 15-o-acetyl esterase (LAE) from *Digitalis lanata* Ehrh. Planta 204, 383–389.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680–685.

Luan, F., Wüst, M., 2002. Differential incorporation of 1-deoxy-D-xylulose into (3S)-linalool and geraniol in grape berry exocarp and mesocarp. Phytochemistry 60, 451–459.

Luthra, R., Luthra, P.M., Kumar, S., 1999. Redefined role of mevalonate-isoprenoid pathway in terpenoid biosynthesis in higher plants. Curr. Sci. 76, 133–135.

Luthra, R., Sangwan, R.S., Sangwan, N.S., 1993. Utilization of exogenously supplied primary precursors for essential oil synthesis in *Cymbopogon* species. Biol. Plant 35, 473–476.

Mahmoud, S.S., Croteau, R., 2002. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends Plant Sci. 7, 366–373.

McCaskill, D., Gershenzon, J., Croteau, R., 1992. Morphology and monoterpene biosynthetic capabilities of secretory cell clusters isolated from glandular trichomes of peppermint (*Mentha piperita* L.). Planta 187, 445–454.

McGarvey, D.J., Croteau, R., 1995. Terpenoid metabolism. Plant Cell 7, 1015–1026.

Preiss, U., Wallnofer, P.R., Engelhardt, G., 1988. Partial purification and properties of an esterase from tomato cell suspension cultures hydrolyzing the pyrethroid insecticide cyfluthrin. Pest. Sci. 23, 13–24.

Rohmer, M., 1999. The discovery of a mevalonate independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat. Prod. Rep 16, 565–574.

Sangwan, N.S., Sangwan, R.S., Luthra, R., Thakur, R.S., 1993a. Geraniol dehydrogenase: a determinant of essential oil quality in lemongrass. Planta Med. 59, 168–170.

Sangwan, R.S., Sangwan, N.S., Luthra, R., 1993b. Metabolism of acyclic monoterpenes: Partial purification and properties of geraniol dehydrogenase from lemongrass (*Cymbopogon flexuosus*). J. Plant Physiol 142, 129–134.

Singh, N., Luthra, R., 1988. Sucrose metabolism and essential oil accumulation during lemongrass (*Cymbopogon flexuosus* Stapf.) leaf development. Plant Sci. 57, 127–133.

Singh, N., Luthra, R., Sangwan, R.S., 1989a. Effect of leaf position and age on the essential oil quantity and quality in lemongrass (*Cymbopogon flexuosus* Stapf.). Planta Med. 55, 254–256.

Singh, N., Luthra, R., Sangwan, R.S., Thakur, R.S., 1989b. Metabolism of monoterpenoids in aromatic plants. Curr. Res. Med. Arom. Plants 11, 174–197.

Singh, N., Luthra, R., Sangwan, R.S., 1990. Oxidative pathways and essential oil biosynthesis in the developing lemongrass (*Cymbopogon flexuosus*) leaf. Plant Physiol. Biochem. 28, 703–710.

Singh, N., Luthra, R., Sangwan, R.S., 1991. Mobilization of starch and essential oil biogenesis during leaf ontogeny of lemongrass (*Cymbopogon flexuosus* Stapf.). Plant Cell Physiol 32, 803–811.

Stuhlfelder, C., Lottspeich, F., Mueller, M.J., 2002. Purification and partial amino acid sequences of an esterase from tomato. Phytochemistry 60, 233–240.

Wise, M.L., Croteau, R., 1999. Monoterpene biosynthesis. In: Cane, D.E. (Ed.), Comprehensive Natural Products Chemistry, vol. 2. Isoprenoids Including Carotenoids and Steroids. Elsevier Science, Oxford, UK, pp. 97–153.