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Inhibitors of Essential Oil Biosynthesis in *Cymbopogon flexuosus* Nees ex. Steud. Mutant cv. GRL-1 Leaves

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Abstract: We studied the effect of metabolic inhibitors on essential oil biosynthesis in terms of change in rate of sodium [2-^{14}C]-acetate (activity 0.1 mCi, specific activity 34.51 mCi mmole^{-1}) incorporation into essential oil in *Cymbopogon flexuosus* (Ness ex Steud) Wats mutant cv. GRL-1 leaves. Out of seven metabolic inhibitors tested, six severely inhibited the label incorporation into essential oil and in particular with their higher concentration. The order of effectiveness of inhibitors expressed as percent inhibition was as follows: sodium arsenate (74.4%) > miconazole (64.35%) > sodium fluoride and sodium borate (47.0%) > 2, 4-di-nitrophenol (DNP) (24.0%). Essential oil biosynthesis, however, could not be inhibited following the mevastatin treatment. The study revealed that metabolic inhibitors interrupted the supply of precursors and co-factors like NADPH, ATP etc. required for cytosolic acetate-mevalonate pathway of essential oil biosynthesis. However, studies with mevastatin clearly supported that besides acetyl Co-A, photosynthetic metabolites, ATP, NADPH, other carbon sources are also utilized for essential oil biosynthesis.

Key words: Sodium [2-^{14}C] acetate, geranyl acetate, geraniol, HMG-CoA reductase, metabolic inhibitors, mevastatin

INTRODUCTION

Essential oils are biosynthesized either through classical acetate/MVA pathway or alternative nonmevalonate (pyruvate/triose-phosphate) pathway (Luthra *et al*., 1999; Liang *et al*., 2002; Rodriguez-Concepcion and Boronat, 2002; Eisenreich *et al*., 2004). Essential oil and terpenoid biosynthesis is presumably, linked to primary metabolism by the rates with which the substrate can branch off from the primary pathways and funnel into secondary biosynthetic routes (Singh *et al*., 1990). Generation of acetyl-CoA, ATP and reducing power, required to drive the isoprenoid biosynthesis, depends upon the activity of oxidative pathways (Baker, 1985). The relationship between primary and secondary metabolic pathways was studied in lemongrass which revealed that metabolism of sucrose and mobilization of starch was most rapid in immature leaves, ensuring an efficient supply of carbon to the growing leaf for various biosynthetic purposes, including terpenoid synthesis (Singh *et al*., 1991). However, in mentha, ^{14}C-fructose, is reported to be a most uptaken sugar for monoterpene (-)-carvone biosynthesis (Maffei *et al*., 2001). Singh *et al*. (1990) in *C. flexuosus* have studied the effect of metabolic inhibitors on essential oil biogenesis and established the active involvement of oxidative pathways in essential oil biosynthesis.

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The interest in compounds interfering with isoprenoid biosynthesis or related processes is increasing owing to their importance in regulation of metabolites. Mevinolin an inhibitor of HMGCoA-reductase (Bach and Lichtenthaler, 1983) and squalen synthase inhibitors (Eisele *et al*., 1997) are in use to regulate the level of cholesterol. The present study was undertaken to investigate the effect of metabolic inhibitor on essential oil biosynthesis and to explore the relationship of these secondary pathways with primary metabolic pathways. Also, mevastatin was used if the formation of monoterpene essential oil had been dependent or independent of the cytosolic acetate-mevalonate pathway. Essential oil biosynthesis was severely affected in presence of all the inhibitors except mevastatin. The results presented here clearly support the idea that essential oil is biosynthesized through cytosolic acetate-mevalonate pathway and is dependent on oxidative (primary) pathways for precursors and co-factors. Contrarily, formation of essential oil in presence of mevastatin suggested the role of other than acetate-mevalonate pathways for essential oil biosynthesis.

MATERIALS AND METHODS

Plant Material

Cymbopogon flexuosus (Ness ex Steud) Wats mutant cv. GRl-1 (geraniol rich) plants were grown in the experimental plot of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India during February-March, 2001. Leaf (slips) cuttings were collected after 15 days of leaf emergence and used for incorporation studies.

Incubation with Sodium [2-^{14}C]-Acetate *in vivo*

In vivo tracer study was performed as described earlier (Singh *et al*., 1990). Leaves detached from 15 day old lemongrass tiller were transferred to test tubes and fed with aqueous solution containing sodium [2-^{14}C]-acetate (activity 0.1 mCi, specific activity 34.51 mCi mmole^{-1}) of strength 5 μCi μmole^{-1} and metabolic inhibitors viz., sodium fluoride, sodium borate, sodium arsenate (concentration 2 and 5 mM), 2,4-dinitrophenol (0.1 and 0.2 mM), miconazole (1 and 2 μg mL^{-1}) and mevastatin (10 and 20 μmol). Leaves were also fed with [2-^{14}C]-acetate without metabolic inhibitors as control. After uptake of the labeled solution, the tubes were kept filled with half-strength Hoagland solution and incubated for 24 h. Leaves were then chopped into small pieces and hydro-distilled using mini-Clevenger's apparatus for essential oil isolation. LKB Wallace 1409 liquid scintillation counter, using PPO-POPOP-toluene cocktail as scintillant analyzed an aliquot of essential oil. Another aliquot of the essential oil was then subjected to thin layer chromatographic separation using toluene: ethyl acetate (96:4, v/v) on silica gel G plates. [^{14}C] geranyl acetate and geraniol spots were scrapped off and analyzed by liquid scintillation counter. The purity of the geranyl acetate and geraniol fractions was confirmed by gas chromatographic analysis. Geraniol and acetate moieties obtained after hydrolyzing geranyl acetate with 10% ethanolic KOH also analyzed by a liquid scintillation counter, as above.

RESULTS

Sodium [2-^{14}C]-Acetate Incorporation with Metabolic Inhibitors

The effect of metabolic inhibitors on essential oil biosynthesis was examined in terms of changes in the level of sodium [2-^{14}C] acetate incorporation into essential oil in 15 day old leaves in presence of these inhibitors (Table 1). In general, all the metabolic inhibitors tested inhibited the sodium [2-^{14}C]-acetate incorporation (pmol/10 leaves) into essential oil from 13 to 75% and in particular with their higher concentration. Mevstatin, however, not inhibited the label incorporation into essential oil. The incorporation (pmol/10 leaves) pattern in geranyl acetate was similar to that of essential oil. In geraniol

Table 1: Effect of metabolic inhibitors on essential oil biosynthesis in *C. flexuosus* mutant cv. GRL-1 leaves. In each treatment, the essential oil was isolated from 15 day old leaves (six numbers) fed with an aqueous solution of [2-^{14}C] acetate (activity 0.1 mCi, specific activity 34.51 mCi mmole^{-1}) of strength 5 μCi μmole^{-1} with or without the inhibitors for 24 h

	[2-^{14}C] acetate incorporated (pmol/10 leaves)			
Treatments	Essential oil	Geranyl acetate	Geraniol	Total geraniol
Acetate (control)	164	97.00	30.00	99.39
Acetate+sodium fluoride (2 mM)	131	72.00	30.00	30.04
(5 mM)	88	36.72	25.00	25.54
Acetate+sodium arsenate (2 mM)	123	42.00	42.00	42.60
(5 mM)	42	16.00	13.00	13.21
Acetate+sodium borate (2 mM)	110	41.00	36.00	36.59
(5 mM)	90	29.00	31.00	31.42
Acetate+dinitrophenol (0.1 mM)	162	60.00	62.00	63.00
(0.2 mM)	125	46.25	34.00	34.72
Acetate+miconazole (1 μg)	152	65.36	43.00	43.86
(2 μg)	58	31.90	14.00	14.41
Acetate+mevastatin (10 μmol)	235	129.72	57.00	58.63
(20 μmol)	277	88.32	88.32	89.45

Table 2: Relative percent distribution of oil incorporated radioactivity into major essential oil constituent viz., geranyl acetate and geraniol. Fifteen day old leaves (six numbers) were incubated with 5 μCi μmole^{-1} of [2-^{14}C] acetate (activity 0.1 mCi, specific activity 34.51 mCi mmole^{-1}) with or without the inhibitors for 24 h

	Distribution of oil incorporated radioactivity (relative %)		
Treatments	Geranyl acetate	Geraniol	Unknown constituents
Acetate (control)	58.49	18.00	23.46
Acetate+sodium fluoride (2 mM)	55.00	23.00	22.00
(5 mM)	41.00	28.00	31.00
Acetate+sodium arsenate (2 mM)	34.00	34.00	32.00
(5 mM)	36.00	29.00	35.00
Acetate+sodium borate (2 mM)	37.00	33.00	30.00
(5 mM)	32.00	34.00	34.00
Acetate+dinitrophenol (0.1 mM)	37.00	38.00	25.00
(0.2 mM)	37.00	27.00	36.00
Acetate+miconazole (1 μg)	43.00	28.00	29.00
(2 μg)	55.00	24.00	21.00
Acetate+mevastatin (10 μmol)	55.00	24.00	21.00
(20 μmol)	32.00	32.00	36.00

the incorporation pattern varied with inhibitors (Table 1). Geraniol biosynthetic capacity of the leaves, expressed as sum of the label present into free geraniol and bound geraniol (as part of geranyl acetate) severely (from 55 to 87%) reduced in the presence these inhibitors (Table 1). Mevastatin inhibitor showed the weak effect on the geraniol biosynthesis (Table 1). Sodium arsenate (5 mM) reduced the incorporation of sodium [2-^{14}C]-acetate into essential oil by 74.4%. Miconazole caused 66% inhibition in label incorporation. Sodium fluoride and sodium borate (5 mM) caused almost 47% inhibitions. 2,4-di nitrophenol DNP) showed weak inhibitory (24.0%) effect on label incorporation. Only sodium borate was found to quite effective at low concentration (2 mM) among the various inhibitors causing 33% reduction in oil incorporated radioactivity.

Distribution of Sodium [2-^{14}C]-Acetate into Major Essential Oil Constituents

The pattern of distribution of radioactivity into major (geranyl acetate and geraniol) and unidentified constituents in presence of all the inhibitors was similar to that of control (without inhibitors). In presence of inhibitors, the radioactivity (relative %) recovered in geranyl acetate was weak as compared to that of geraniol. Marked fluctuation (20 to 39%) in relative distribution of label into unknown constituent was observed in presence of inhibitors (Table 2). The pattern of

Table 3: Relative percent distribution of radioactivity into geraniol and acetate moiety of geranyl acetate and in free and bound geraniol (as part of geranyl acetate) in presence of metabolic inhibitors

	Relative % distribution of radioactivity			
	Geranyl acetate		Total geraniol	
Treatments	Geraniol +	Acetate	Free geraniol +	Bound geraniol
Acetate (control)	71.54	28.46	30.18	69.82
Acetate+sodium fluoride (2 mM)	1.17	98.83	97.27	2.73
(5 mM)	1.49	98.51	97.86	2.14
Acetate+sodium arsenate (2 mM)	1.43	98.57	98.59	1.41
(5 mM)	1.33	98.67	98.41	1.59
Acetate+sodium borate (2 mM)	1.44	98.56	98.39	1.61
(5 mM)	1.44	98.56	98.66	1.34
Acetate+dinitrophenol (0.1 mM)	1.68	98.32	98.41	1.59
(0.2 mM)	1.56	98.44	97.93	2.07
Acetate+miconazole (1 μg)	1.32	98.68	98.04	1.96
(2 μg)	1.28	98.72	97.15	2.85
Acetate+mevastatin (10 μmol)	1.47	98.53	97.90	2.10
(20 μmol)	1.12	98.88	98.37	1.63

radioactivity distribution (relative %) into geraniol and acetate moiety of geranyl acetate was just opposite to that of control (without inhibitors). Majority (97-99%) of the geranyl acetate incorporated radioactivity was detected into acetate moiety, whereas, very low 1-2% into the bound geraniol (as part of geranyl acetate) in presence of all the inhibitors. Relative percent distribution of label present in free geraniol and bound geraniol (as part of geranyl acetate) followed the similar trend to those observed in geraniol and acetate moiety with majority (96-99%) of label observed in free geraniol as compared to that of bound geraniol (Table 3).

DISCUSSION

In the present article the effect of metabolic inhibitors on essential oil biosynthesis is investigated using sodium [2-^{14}C]-acetate. Earlier, [2-^{14}C]-acetate incorporation studies in lemongrass have shown that only young and rapidly expanding leaves have the capacity to synthesize and accumulate essential oil (Singh *et al*., 1989, 1990 and 1991; Ganjewala and Luthra, 2007). Hence, the study was performed in immature 15 day old leaves. The observed severe reduction in [2-^{14}C]-acetate incorporation into essential in presence of these metabolic inhibitors was due to the blockage of the oxidative pathways. These results are analogous to those of earlier reported in *C. flexuosus* and suggested the tight integration of essential oil biosynthesis with the primary metabolic pathways (Singh *et al*., 1990). Analogous inhibitions of fatty acid biosynthesis by these inhibitors are also reported in developing seeds of soyabean (Yamada *et al*., 1974) sunflower (Monga *et al*., 1983) and nasturtium (Pollard and Stumpf, 1980). Methyl viologen and cerulenin have recently been reported as inhibitors of fatty acid biosynthesis in sunflower (Pleite *et al*., 2006).

Essential oils (monoterpenes) are derived from geranyl diphosphate (GPP). Perez *et al*. (1980) have reported the role allylic phosphatase activities from flavedo of *Citrus sinensis* in formation of alcohols (geraniol or fernasol) from their corresponding allylic pyrophosphates (GPP or FPP). However, the formation of allylic alcohol (geraniol) by cell free system was depleted following sodium fluoride treatment due to hindering of the allylic phosphatase activities. Hence, in lemongrass mutant cv. GRL-1, it seems that sodium fluoride might have interfered hydrolysis of GDP and glycolytic pathway which resulted in decrease in essential oil biosynthesis similar to those reported earlier (Perez *et al*., 1980; Goodwin and Mercer, 1983; Piazza and Holzwarth, 1989). The effect of sodium arsenate on essential oil biosynthesis was due to interference of substrate level (glycoltic) and oxidative phosphorylations. The decrease in essential oil biosynthesis in presence of sodium borate must be due

to NADPH depletion. In this case the inhibition is likely to result from interference of dehydrogenases, catalyzing production of NADPH from 6-phosphogluconic acid (Dugger, 1983; Piazza and Holzwarth, 1989). Dinitrophenol is reported to be a un-coupler of oxidative phosphorylation. The lack of available ATP for mevalonate kinase and mevalonate pyrophosphate decarboxylase of acetate-mevalonate pathway following 2,4-di-nitrophenol treatment leads to decrease in essential oil biosynthesis (Singh *et al*., 1990). The decrease in essential oil biosynthesis by miconazole may likely to results from interference of cytochrome function which in turn interrupted isoprenoid biosynthesis (Heit and Riviere, 1985).

Mevastatin is reported to be an inhibitor of the cytosolic IPP biosynthesis (Bach, 1987). In lemongrass mutant cv. GRL-1 mevastatin showed no effect on the incorporation of sodium [2-^{14}C] acetate into essential oil. According to non-mevalonate pathway acetate is not direct precursor of monoterpene biosynthesis. Therefore, the result could merely represent the enhanced level of acetate, which is not used for sterol biosynthesis and which is then used to form precursors of the non-mevalonate pathways via unspecific metabolization in primary metabolism as in biosynthesis of marubinn (Knoss and Reuter, 1998). However, we could not study the change in HMG-CoA reductase activity as a consequence of mevalonate treatment.

Though consistency in the relative percent distribution of radioactivity among the oil constituents (geranyl acetate and geraniol) was observed, but geraniol (free+bound geraniol) biosynthetic capacity of the leaves reduced substantially in presence of all the inhibitors. However, mevastatin showed weak effect on geraniol biosynthesis. In presence of all the inhibitors tested, the radioactivity distribution pattern in geraniol and acetate moiety of geranyl acetate and free to bound geraniol was similar but opposite to that of acetate (without inhibitors).

In conclusion, in lemongrass mutant cv. GRL-1 the significant diminution in essential oil biosynthesis in presence of all the metabolic inhibitors (except mevastatin) is likely to result from interference of cytosolic acetate-mevalonate pathway which is dependent on primary metabolic pathways for precursor and co-factors. However, if the formation of monoterpene essential oil had been dependent on the cytosolic acetate-mevalonate pathway, the formation of essential oil should have been fully inhibited by mevastatin. This clearly support that besides acetyl Co-A, photosynthetic metabolite, ATP, NADPH, other carbon source are also utilized for essential oil biosynthesis.

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