Differential Accumulation of Dimethylallyl Diphosphate in Leaves and Needles of Isoprene- and Methylbutenol-Emitting and Nonemitting Species[1]

Todd N. Rosenstiel, Alison J. Fisher[2], Ray Fall, and Russell K. Monson*

Departments of Environmental, Population, and Organismic Biology (T.N.R., R.K.M.) and Chemistry and Biochemistry (A.J.F., R.F.) and Cooperative Institute for Research in Environmental Sciences (R.F., R.K.M.), University of Colorado, Boulder, Colorado 80309–0334

The biosynthesis and emission of volatile plant terpenoids, such as isoprene and methylbutenol (MBO), depend on the chloroplastic production of dimethylallyl diphosphate (DMAPP). To date, it has been difficult to study the relationship of cellular DMAPP levels to emission of these volatiles because of the lack of a sensitive assay for DMAPP in plant tissues. Using a recent DMAPP assay developed in our laboratories, we report that species with the highest potential for isoprene and MBO production also exhibit elevated light-dependent DMAPP production, ranging from 110% to 1,063%. Even species that do not produce significant amounts of volatile terpenoids, however, exhibit some potential for light-dependent production of DMAPP. We used a nonaqueous fractionation technique to determine the intracellular distribution of DMAPP in isoprene-emitting cottonwood (*Populus deltoides*) leaves; approximately 65% to 70% of the DMAPP recovered at midday occurred in the chloroplasts, indicating that most of the light-dependent production of DMAPP was chloroplastic in origin. The midday concentration of chloroplastic DMAPP in cottonwood leaves is estimated to be 0.13 to 3.0 mM, which is consistent with the relatively high K_ms that have been reported for isoprene synthases (0.5–8 mM). The results provide support for the hypothesis that the light dependence of isoprene and MBO emissions is in part due to controls over DMAPP production.

A wide array of volatile organic compounds (VOCs) are emitted into the atmosphere by the leaves of many plant species (Graedel, 1979; Lerdau et al., 1997; Kesselmeier and Staudt, 1999; Kreuzwieser et al., 1999). Among the biogenic VOCs studied to date, isoprene (2-methyl-1,3-butadiene) is quantitatively the most important, with as much as 500 Tg year^{-1} estimated to be emitted globally from vegetation (Guenther et al., 1995), exerting profound effects on atmospheric chemistry (Monson and Holland, 2001). In the presence of nitrogen oxides and sunlight, isoprene oxidation can lead to the production of tropospheric ozone (Trainer et al., 1987; Chameides et al., 1988). Not all plants emit isoprene. Most that do are woody in growth habit and are represented by North American species of oaks (*Quercus* spp.), willows (*Salix* spp.), poplars (*Populus* spp.), and spruce (*Abies* spp.; Harley et al., 1999).

Methylbutenol (MBO; 2-methyl-3-buten-2-ol) is a C_5 terpenoid, similar to isoprene, that is emitted by several pine species native to the western United States, including ponderosa (*Pinus ponderosa*), lodgepole (*Pinus contorta*), and gray pine (*Pinus sabiniana*; Harley et al., 1998). Emissions of MBO from pine needles are dependent on both photosynthetic photon flux density (PPFD) and temperature (Harley et al., 1998), showing behavior that is similar to leaf emissions of isoprene (Monson and Fall, 1989; Loreto and Sharkey, 1990). Like isoprene, MBO emissions from forests represent a significant source of reactive carbon to the atmosphere (Harley et al., 1998). Despite the important influence of isoprene and MBO emissions on atmospheric chemistry, the biological functions of these VOCs remains open to debate (Logan et al., 2000; Sharkey and Yeh, 2001).

Two lines of evidence suggest that isoprene and MBO arise from similar biochemical pathways in plants. First, both of these C_5 hemiterpenes are formed via the recently discovered non-mevalonate, 1-deoxyxylulose 5-phosphate/2-C-methylerythritol 4-phosphate (DOXP/MEP) pathway of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) formation (Lichtenthaler et al., 1997; Zeidler and Lichtenthaler, 2001). This pathway utilizes pyruvate and glyceraldehyde-3-phosphate for the synthesis of these C_5 prenyl diphosphates (Lichtenthaler, 1999). All plastidic terpenoids studied thus far, including isoprene, MBO, carotenoids, and the phytol

[1] This work was supported by the National Science Foundation (grant nos. BIR–9413218 to R.K.M. and ATM–9633285 to R.F.), by the Department of Energy (grant no. DE–FG03–97ER20274 to R.F.), and by the Cooperative Institute for Research in Environmental Science (graduate fellowships to A.J.F. and T.N.R.).

[2] Present address: Department of Chemistry, Norwich University, Northfield, VT 05663.

* Corresponding author; e-mail russell.monson@colorado.edu; fax 303–492–8699.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.002717.

side chain of chlorophyll (Chl), are formed via the DOXP/MEP pathway (Lichtenthaler, 1999). Second, Fisher et al. (2000) detected and partially purified an MBO synthase from gray pine needles that catalyzes the conversion of DMAPP to MBO. This reaction is similar to the formation of isoprene via isoprene synthase (Silver and Fall, 1995), except that a putative allylic carbocation intermediate is hydrated rather than undergoing proton extraction and rearrangement. These reactions are shown in Figure 1.

The regulatory mechanisms controlling isoprene and MBO emission are not completely understood, although both short- and long-term variation in isoprene emission appears to depend on the activity of isoprene synthase (Monson et al., 1992; Kuzma and Fall, 1993; Schnitzler et al., 1997). Diurnal fluctuations in isoprene emission rates are generally well correlated with PPFD (Sharkey and Loreto, 1993) and leaf temperature (Monson et al., 1992). The PPFD dependence of isoprene emission has been attributed to control by photosynthetic electron transport (Niinemets et al., 1999; Zimmer et al., 2000), as well as direct light activation of isoprene synthase (Wildermuth and Fall, 1996; Fall and Wildermuth, 1998). Isoprene synthases from aspen (*Populus tremuloides*), velvet bean (*Mucuna* sp.), and oak, when assayed in vitro, have temperature response profiles that are generally similar to that for corresponding leaves (Monson et al., 1992; Schnitzler et al., 1996). Long-term and seasonal variation in isoprene emission has been reported (Monson et al., 1994), and in at least one case, the seasonality of emission was related to seasonal variation in extractable isoprene synthase activity (Schnitzler et al., 1997).

Although much of our current understanding of the regulation of leaf level isoprene emissions has been interpreted with respect to the activity of isoprene synthase, it has been suggested that the regulation of isoprene emission may also, in part, depend on the availability of DMAPP substrate (Wildermuth and Fall, 1996; Fall and Wildermuth, 1998; Sharkey and Yeh, 2001). Presumably, activity of the DOXP/MEP pathway must be regulated to accommodate the large changes in isoprene and MBO emission that are known to occur. Preliminary reports have suggested that transcripts of two enzymes of the DOXP/MEP pathway (deoxyxylulose synthase and deoxyxylulose reductoisomerase) are induced and accumulate in leaves of an isoprene emitter when switched to high temperature (Sharkey and Yeh, 2001).

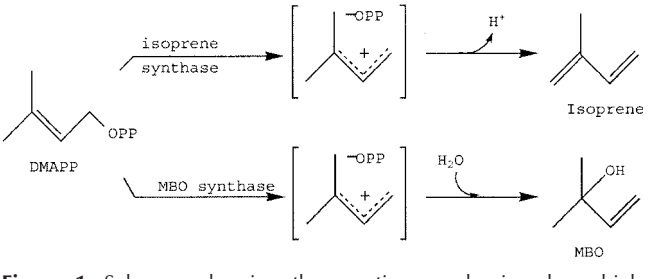

Figure 1. Schemes showing the reaction mechanism by which DMAPP is catalytically converted to isoprene or MBO by isoprene synthase or MBO synthase.

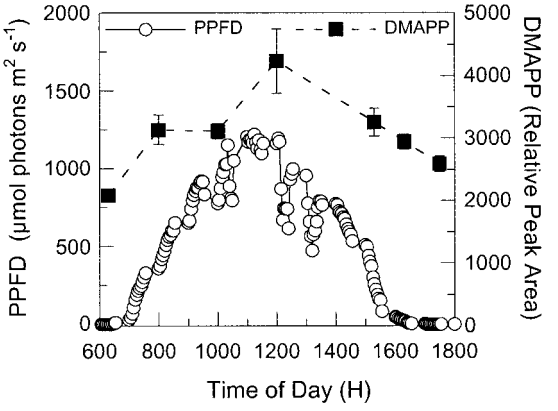

Figure 2. Diurnal variation in the PPFD (400–700 nm) and whole-leaf DMAPP concentration for leaves of potted cottonwood that were grown in the University of Colorado greenhouse. The DMAPP symbols represent the mean ± SE of age-matched leaves from five replicate plants.

Recently, we developed a method to quantify DMAPP, an end product of the DOXP/MEP pathway, in biological samples (Fisher et al., 2001). Using this technique, we set out to determine if patterns of accumulation of DMAPP in leaves and needles vary among isoprene- and MBO-emitting and nonemitting plant species. In this study we provide the first evidence for differences in the regulation of DMAPP levels in leaves. Because production of DMAPP is compartmentalized in plant cells (Lichtenthaler et al., 1997), occurring separately in both the cytosol and plastids, we present evidence for the localization of DMAPP in cottonwood (*Populus deltoides*) leaves.

RESULTS

Diurnal Time Course of DMAPP Content

In a past study, we developed methods for quantifying DMAPP in leaf and needle tissues with recovery greater than 95%. Using this method, we observed large diurnal increases in relative DMAPP content in leaves of the isoprene-emitting species cottonwood when sampled at predawn and midday (Fisher et al., 2001). Prompted by these results, in the current study, we measured DMAPP throughout the day in leaves from several trees of cottonwood exposed to a natural photoperiod (Fig. 2). The relative content of DMAPP in whole-leaf preparations changed through the day, and appeared to correspond with changes in incident PPFD. Quantifiable levels of DMAPP were clearly present in leaves at the beginning and end of the photoperiod. By midday, relative DMAPP content increased nearly 2.5-fold, returning to near predawn levels by dusk.

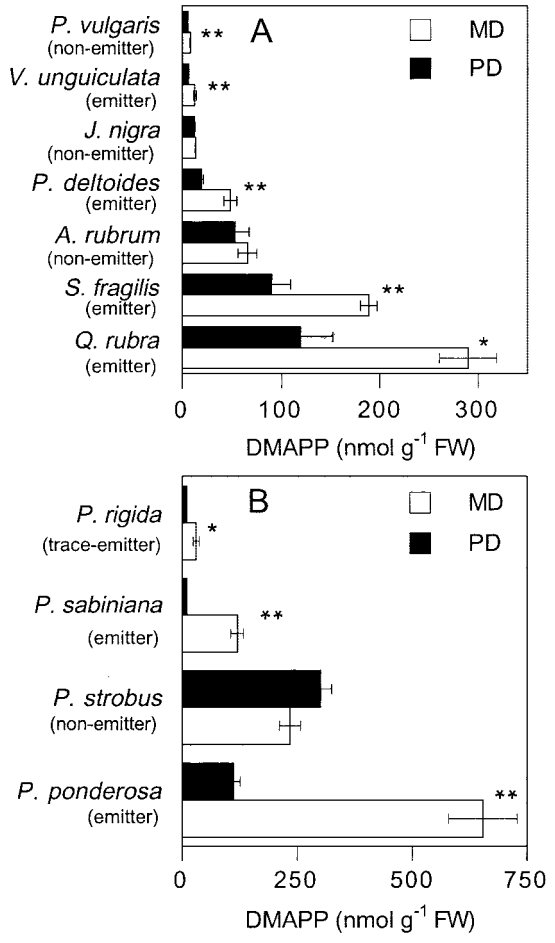

Figure 3. The midday (MD) and predawn (PD) whole-leaf DMAPP concentrations for several isoprene-emitting species (A) and MBO-emitting species (B) growing on the University of Colorado campus or in the greenhouses. Horizontal bars represent the mean ± SE of leaves from three to five replicate plants. A single asterisk by each pair of values represents predawn and midday differences at the significance level of $P < 0.01$ and a double asterisk represents predawn and midday differences at the significance level of $P < 0.05$.

Survey of DMAPP Content in Leaves of Isoprene- and MBO-Emitting and Nonemitting Species

We next quantified DMAPP content, at two times of day (predawn and midday), in leaves from seven species growing on the campus and in the greenhouses of the University of Colorado (Fig. 3A). The species chosen represented a mix of known isoprene-emitting and nonemitting species. We were interested in determining if the large diurnal increases in DMAPP content that were observed in cottonwood were a general phenomenon or a specific characteristic of isoprene-emitting plants. All leaves examined contained quantifiable levels of DMAPP at both predawn and midday. However, the absolute content of DMAPP varied markedly among the species examined, with isoprene emitters generally exhibiting the highest levels of DMAPP. The nonemitter common pole bean (*Phaseolus vulgaris*) had the lowest DMAPP content at both predawn and midday (5.37 ± 0.18 and 7.64 ± 0.35 nmol g^{-1} fresh weight, respectively). DMAPP levels at predawn and midday in the weak isoprene emitter cowpea (*Vigna unguiculata*) were similarly low (5.92 ± 0.46 and 12.43 ± 1.31 nmol g^{-1} fresh weight, respectively). In contrast, leaves from the strong isoprene emitter red oak (*Quercus rubra*) contained the highest levels of DMAPP at predawn and midday (119.4 ± 32.65 and 290 ± 29.42 nmol g^{-1} fresh weight, respectively). All of the isoprene emitters tested, and the nonemitting common pole bean, showed significant increases in whole-leaf DMAPP content from predawn to midday ($P < 0.05$). The two nonemitters common walnut (*Juglans nigra*) and red maple (*Acer rubrum*) exhibited no significant diurnal changes in DMAPP content ($P = 0.73$ and 0.48, respectively). Although absolute levels of DMAPP varied significantly among the isoprene-emitting and nonemitting species, isoprene-emitting species displayed a 110% to 149% increase in DMAPP content from predawn to midday, whereas nonemitters exhibited smaller increases from 9% to 42% (Table I).

To determine if large diurnal changes in DMAPP content were also characteristic of MBO-emitting species, a similar analysis was performed on needles collected from trees of four pine species growing in a greenhouse at the University of Colorado (Fig. 3B). The DMAPP content of needles varied significantly among the species examined. Similar to the results for isoprene emitters, all of the pines tested contained quantifiable levels of DMAPP at predawn, with the nonemitting eastern white pine containing the greatest levels (300.7 ± 22.40 nmol g^{-1} fresh weight). Needles collected at midday from the MBO emitter, ponderosa, displayed the greatest DMAPP contents of all pine species (654.4 ± 74.44 nmol g^{-1} fresh weight). All of the MBO-emitting species exhibited significant increases in whole-needle DMAPP content when comparing predawn and midday measurements ($P < 0.05$). In contrast, needles from the nonemitter eastern white pine displayed no significant diurnal changes in DMAPP content ($P = 0.10$). Despite the large variation in DMAPP content among

Table I. *Summary of percent increases in DMAPP content from predawn to midday*

Note that data for this summary are given in Figure 2 and represent the average percent increase of three to five individual leaf samples. Both isoprene and MBO emitters are included in the "emitting species" category.

Nonemitting Species		Emitting Species	
	%		%
Eastern white pine (*Pinus strobus*)	−22	*Salix fragilis*	110
Common walnut	9	Cowpea	110
Red maple	23	Red oak	143
Common pole bean	42	Cottonwood	149
		Pinus rigida	184
		Ponderosa pine	489
		Gray pine	1,063

the four pine species tested, MBO-emitting species exhibited pronounced increases in DMAPP content predawn to midday (110%–1,063%), whereas the nonemitting species eastern white pine did not (Table I).

In general, we expressed DMAPP content on a fresh weight basis. To determine if the basis for expression alters the interpretation of results, we performed additional analyses of leaf and needle tissue, expressing DMAPP content on several common bases, including nmol g^{-1} fresh weight, nmol g^{-1} dry weight, nmol mg^{-1} Chl and nmol mg^{-1} protein (Table II). Irrespective of the basis for expression, the midday content of DMAPP varied consistently among the species examined. Among fully expanded leaves and needles, the absolute content of DMAPP was highest in the nonemitting eastern white pine. In contrast, young leaves of cottonwood contained the lowest levels of DMAPP. Similarly, young leaves of cottonwood contained significantly less DMAPP for a given basis of expression than a similarly orientated mature leaf ($P < 0.05$).

Cellular Localization of DMAPP

Next, we determined the relative intracellular localization of DMAPP in leaves of the isoprene emitter cottonwood harvested at midday. Leaves were fractionated using a nonaqueous fractionation technique (Gerhardt and Heldt, 1984), and DMAPP levels in each fraction were subsequently measured. A typical density gradient with associated distributions of markers and DMAPP is shown in Figure 4. Chl, the chloroplast marker, occurred in the upper, lightest, fractions; the greatest percentage of vacuolar marker (α-mannosidase) occurred in the lower, heaviest, fractions; and the cytosolic marker (phospho*enol*-pyruvate carboxylase) generally partitioned toward the middle of the gradient. This relative distribution of compartment markers is typical for plant leaves after centrifugation in nonaqueous gradients (Gerhardt and Heldt, 1984; Sharkey and Vanderveer, 1989; Stitt et al., 1989; Moore et al., 1995). In comparison with the distribution of markers, DMAPP was predominately associated with Chl. In every case, approximately 70% of the DMAPP recovered from the entire gradient was partitioned into the lightest two fractions, and it was always visually associated with Chl. The correlation coefficient between percent DMAPP and percent Chl in numerous gradients was generally greater than 0.98 (data not shown).

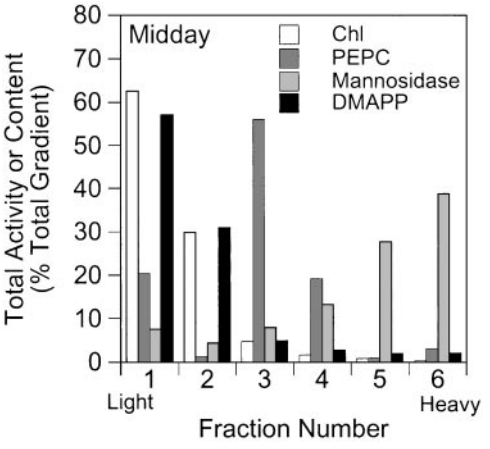

Figure 4. The partitioning of various markers and DMAPP within the nonaqueous fractions that were used. Data are from a single gradient representative of five different experiments.

The activities or amounts of markers and analytes in the gradient fractions were examined by graphical analyses to determine relative distributions between the cytosol and chloroplasts. Using this two-compartment analysis technique, we determined that approximately 65% to 70% of the DMAPP in leaves of cottonwood harvested at midday occurs in the chloroplast (Table III). Attempts were made to perform similar analyses on predawn leaves. Unfortunately, we were unsuccessful in measuring active phospho*enol*pyruvate carboxylase from these preparations. However, several attempts did provide anecdotal evidence that the greatest fraction of predawn DMAPP was recovered from fractions lower in the gradient, compared with midday DMAPP. However, the lack of a suitable predawn cytosolic marker precludes any further interpretation.

DISCUSSION

The work presented here represents the first detailed measurements of DMAPP in plant leaves. This

Table II. *DMAPP content of leaves of four isoprene- and MBO-emitting and nonemitting species*

Leaves, or needles, of each species were similarly orientated and harvested on the same day in the middle of the photoperiod; incident PPFD = 1,650 μE. Values are means ± SD, n = 4 to 6.

Species	DMAPP				
	μmol m⁻²	*nmol g fresh wt⁻¹*	*nmol g dry wt⁻¹*	*nmol mg Chl⁻¹*[a]	*nmol mg protein⁻¹*
Cottonwood (young; isoprene-emitting)	2.16 ± 0.25	13.86 ± 1.56	49.38 ± 5.54	8.07 ± 0.14	0.17 ± 0.15
Cottonwood (isoprene-emitting)	14.23 ± 1.62	91.17 ± 10.36	257.5 ± 29.25	33.97 ± 0.13	1.19 ± 0.12
Common pole bean (nonemitting)	6.28 ± 0.45	40.24 ± 2.9	252.3 ± 18.19	19.28 ± 0.08	0.84 ± 0.18
Eastern white pine (nonemitting)	n.d.[b]	328.6 ± 43.98	851.7 ± 114.0	229.0 ± 0.14	7.43 ± 0.17
Gray pine (MBO-emitting)	n.d.	180.0 ± 10.56	498.3 ± 29.24	106.4 ± 0.11	4.53 ± 0.18

[a] Chl, Total Chl (a + b). [b] n.d., Not determined.

Table III. *Intracellular distribution of DMAPP in illuminated leaves of cottonwood*

Values are presented from two separate experiments. For each experiment, fully exposed leaves were sampled directly into liquid nitrogen at midday, incident PPFD = 1,500 μE. Distributions were calculated according to a two-compartment model as described in "Materials and Methods."

Sample No.	Total DMAPP in Gradient Fractions	Distribution of DMAPP	
		Chloroplast	Cytosol
	nmol	*%*	
1	12.49	65	35
2	10.21	71	29

is of general interest in plant biology because DMAPP is one of the essential precursors of all plant terpenoids. Two key findings are presented. First, we detected a diurnal increase in DMAPP content that was pronounced in leaves of isoprene and MBO emitters, but there was little or no increase in leaves of nonemitters. Second, in isoprene-emitting cottonwood leaves, DMAPP was largely localized in chloroplasts. Both of these observations may be directly related to the biological rationale for light-dependent hemiterpene emission from leaves, a subject of some debate for several decades (Logan et al., 2000; Sharkey and Yeh, 2001), and are of general interest in understanding the regulation of higher terpenoid biosynthesis in leaves.

We first observed large diurnal changes in DMAPP content in leaves of cottonwood while developing a sensitive nonradioactive method for quantifying DMAPP in biological samples (Fisher et al., 2001). In the current study, a more complete characterization revealed that DMAPP levels vary diurnally in a manner that roughly parallels incident PPFD (Fig. 2). This pattern of DMAPP accumulation is remarkably similar to previously reported patterns of isoprene emission versus PPFD (Monson and Fall, 1989; Loreto and Sharkey, 1990; Monson et al., 1992). When additional leaves of isoprene-emitting and nonemitting species were compared, we found that the capacity to emit isoprene was clearly associated with large diurnal variation in DMAPP content (Fig. 3; Table I). Differences in the magnitude of diurnal DMAPP accumulation were also seen in a comparison of MBO-emitting and nonemitting pine species (Fig. 3; Table I), and the differences in accumulation correlate well with known patterns of emission (for estimates of MBO emission rates in these species, see Harley et al., 1998).

There is clearly a tendency for amplification of the capacity for light-dependent DMAPP production in isoprene- and MBO-emitting species. However, the fact that even some nonemitters exhibit diurnal increases in DMAPP suggests that the light-dependent response may be a more general characteristic of plant metabolism. The light-dependent synthesis of IPP and DMAPP could support the production of higher isoprenoids during periods of high photosynthetic activity, including the replacement and repair of damaged Chls, carotenoid molecules, and pigment-protein complexes that occur at high PPFD (Niyogi, 2000).

When midday DMAPP contents were expressed on different measures, and compared in isoprene- and MBO-emitting and nonemitting species, no significant differences in interpretation were found (Table II). However, it was apparent that young (isoprene-emitting) leaves of cottonwood contained significantly less DMAPP on all measures than did similarly orientated fully mature leaves. This may simply reflect a partially depleted pool of free DMAPP as a result of increased carotenoid and Chl biosynthesis during leaf development. It may also represent an overall decrease in the synthesis of DMAPP as a result of the presumed reduction in photosynthetic metabolism. Although the results are preliminary, they suggest that the availability of DMAPP may, in some way, be associated with the ontogenetic delay in isoprene emission that has been observed in previous studies (Kuzma and Fall, 1993).

Cellular Localization of DMAPP

It is now known that DMAPP is produced by two distinct and independent biosynthetic routes within the plant cell; the classic mevalonic acid pathway in the cytosol and the DOXP/MEP pathway of plastids (Lichtenthaler et al., 1997). To interpret the relevance of our measures of DMAPP content for influencing VOC emission, it is necessary to provide an estimate of the relative distribution of DMAPP between these two cellular compartments. We used nonaqueous fractionation as a method for determining the intracellular distribution of DMAPP in leaves of cottonwood (Table III). There are two ways of calculating subcellular metabolite distributions from nonaqueous fractionation data: a two-compartment analysis (Gerhardt and Heldt, 1984) and a three-compartment analysis (Riens et al., 1991). Because DMAPP is generally thought to only occur in the cytosol and chloroplasts, and the presence of phosphorylated intermediates are generally absent from the vacuole (Farré et al., 2001), the two-compartment model is justifiable (see discussion in "Materials and Methods"). Results from our analysis suggest that a significant percentage (65%–71%) of DMAPP measured at midday in cottonwood leaves is associated with the chloroplast. It seems likely that these two distinct pools of DMAPP derive from the two independent pathways for isoprenoid synthesis (Licthenthaler et al., 1997). However, whether the two pathways exchange intermediates is not well understood, although Heintze et al. (1990) and McCaskill and Croteau (1995) have suggested that IPP could be transported across the plastid envelope.

Using the results from the localization experiment, it is possible to derive an estimate of the concentra-

tion of DMAPP in isoprene-emitting chloroplasts. Our measurements reveal that the whole-leaf concentration of DMAPP in isoprene emitters ranges from 2 to 45 μmol m^{-2}. If it is assumed that 60% of DMAPP occurs in the chloroplast, and that the ratio of chloroplast volume to total leaf area is 0.009 L m^{-2} (as determined in *Quercus robur* by Zimmer et al., 2000), then the concentration of chloroplastic DMAPP would range from 0.13 to 3.0 mM. This estimate is consistent with the relatively high K_m values that have been reported for isoprene synthase (0.5–8 mM; Silver and Fall, 1995; Schnitzler et al., 1996) and MBO synthase (5 mM; Fisher et al., 2000). The concentration of chloroplastic DMAPP is similar to those reported for other chloroplastic phosphorylated intermediates, including 3-phosphoglycerate (2.0–4.3 mM), dihydroxyacetone phosphate (0.21–0.32 mM), and Fru-1,6-bisphosphate (0.55–1.04 mM; Winter et al., 1993, 1994; Leidreiter et al., 1995).

When taken together, the large diurnal variation in DMAPP content associated with isoprene and MBO-emission, as well as the localization of DMAPP to the cottonwood chloroplast, suggests that the production of DMAPP may participate in the regulation of isoprene and MBO emission from plant leaves. Previous studies have primarily emphasized the importance of the synthase enzymes in regulating light-dependent VOC emission (Sharkey and Yeh, 2001). However, in at least one study of isoprene emission in oak, Lehning et al. (1999) reported no diurnal changes in extractable isoprene synthase activity despite marked diurnal variation in emission rate, concluding that diurnal variation in leaf temperature could account for observed diurnal changes in isoprene emission rate. However, this analysis depended on the assumption of constant and saturating DMAPP substrate conditions. Results from our study suggest that a constant supply of DMAPP does not occur in isoprene-emitting species during the diurnal cycle. This raises the possibility that some of the diurnal variation in isoprene emission observed by Lehning et al. (1999), and in general the PPFD dependence of isoprene and MBO emission, is due, in part, to variation in DMAPP substrate supply.

Substrate level regulation of isoprenoid biosynthesis is not without precedent. In animals (Edmond and Popjak, 1974), and in some plants (Nes and Bach, 1985; Bach et al., 1999), excess carbon is diverted away from isoprenoid production by the "mevalonate shunt," a series of reactions converting DMAPP to dimethylacrylyl-CoA, an intermediate of Leu metabolism. This shunting of carbon away from isoprenoid biosynthesis has been shown to regulate isoprenoid synthesis in embryonic *Drosophila melanogaster* cells (Havel et al., 1986) and in rat (*Rattus norvegicus*) liver (Marinier et al., 1987). Recently, overexpression of either deoxyxylulose synthase or deoxyxylulose reductoisomerase, two key enzymes in the DOXP/MEP pathway, led to the accumulation of various isoprenoids such as Chls, carotenoids, and abscisic acids in Arabidopsis (Estévez et al., 2001) and an increase in essential oil accumulation in peppermint (*Mentha* × *piperita*; Mahmoud and Croteau, 2001), respectively. These results support the role of substrate level regulation of isoprenoid biosynthesis.

Although our understanding of the regulatory mechanisms controlling isoprene and MBO emission are improving, the ultimate reason(s) for these emissions remains unclear. Several experiments have provided support for the hypothesis that volatile isoprenoids (isoprene, monoterpenes, and other short-chain hydrocarbons) protect leaves from short high-temperature bursts (Sharkey and Yeh, 2001). However, evidence for increased thermotolerance due to isoprene was not found in at least two studies (Logan and Monson, 1999; Logan et al., 1999). Other experiments demonstrate that isoprene protects leaves exposed to high-ozone episodes and therefore isoprene may play an important antioxidative role in plant leaves (Loreto et al., 2001). However, previous studies have suggested that the hydroperoxides produced by the reaction between ozone and isoprene may be as damaging to leaves as ozone alone (Hewitt et al., 1990; Sauer et al., 1999).

An alternative hypothesis to explain the possible advantages of isoprene emission has focused on the regulation of chloroplast metabolism through maintenance of appropriate pool sizes for various metabolites (Logan et al., 2000). Although support for this hypothesis remains correlative in nature, results from this study suggest that the total flux of carbon through the DOXP/MEP pathway can be significant. The conversion of DMAPP to isoprene and MBO, and their emission, may help regulate the size of the midday DMAPP pool, and thereby release previously unavailable phosphate.

Results from this study highlight the need for an enhanced understanding of the role of the DOXP/MEP pathway in regulating the biosynthesis of isoprenoid VOCs. The pronounced diurnal variation in DMAPP associated with isoprene and MBO emission suggests an inherent difference in the regulation of this pathway in species that synthesize isoprene and MBO, compared with nonemitting species. Alteration in the activity of this pathway may result from altered activities of DOXP/MEP enzymes, enhanced supply of pathway precursors, or both. A careful reexamination of the sources of carbon entering the DOXP/MEP pathway will be required to further test the hypothesized role of isoprene emission in metabolic regulation, as well as refine the potential significance of substrate level control of isoprene and MBO emission from leaves and needles. At present, we are developing new experimental systems using intact chloroplasts, which will allow us to test the validity of these ideas.

MATERIALS AND METHODS

Plant Material

Leaves and needles were collected from trees growing on the campus and in the greenhouses of the University of Colorado. Whole leaves were collected directly into liquid N_2 at two times of the day, immediately before sunrise (predawn) and at the middle of the photoperiod (midday). All leaves were fully exposed sun leaves, and were uniformly orientated with respect to incident light and of similar age. Leaves were stored in liquid N_2 until further processing.

Assay and Quantification of DMAPP

DMAPP was quantified as described previously (Fisher et al., 2001). In brief, the levels of DMAPP were determined by measuring the amount of isoprene released into the headspace of a sealed reaction vial upon acidification. Frozen leaves were ground to a fine powder with added liquid N_2 in a mortar and pestle, and 100 mg of the resulting powder was rapidly transferred to a 4.8-mL glass vial containing 500 μL of distilled water. This suspension was immediately acidified by the addition of 500 μL of 8 M H_2SO_4 and sealed with a Teflon-lined septum. Vials were incubated for 1 h at 30°C, after which 1.5 mL of the vial headspace was withdrawn with a gas-tight syringe, and analyzed for isoprene by gas chromatography, described in detail in Fisher et al. (2001). Calibration of the detection system was performed using authentic isoprene standard (Scott Specialty Gases, Longmont, CO). Isoprene production from DMAPP hydrolysis was calibrated using aqueous solutions of DMAPP synthesized and purified as previously described (Davisson et al., 1985). Although isoprene is one of only several hydrolysis products resulting from the acidification of DMAPP, we have found that the production of isoprene is linear from about 2 pmol to 15 nmol DMAPP with this method. Recovery of DMAPP in leaf and needle samples was greater than 95% in all cases. Although needles of MBO-emitting pines contain small amounts of MBO, about 0.06 nmol g^{-1} fresh weight in illuminated ponderosa (*Pinus ponderosa*; Fisher, 2001), and MBO produces isoprene upon acidification (Fisher et al., 2001), these levels are insignificant in the DMAPP determinations.

Nonaqueous Fractionation

The nonaqueous fractionation procedures were similar to those reported elsewhere (Gerhardt and Heldt, 1984; Sharkey and Vanderveer, 1989). Sun-exposed cottonwood (*Populus deltoides*) leaves were collected directly into liquid N_2 at midday. After removing the major veins, 5 g of leaves was ground in liquid N_2 using a mortar and pestle. Powdered material was transferred to cold lyophilization jars and immediately placed on a freeze-drying apparatus (Labconco, Kansas City, MO), with a collector temperature of −80°C. The jars were kept in liquid N_2 until the air pressure was reduced to ≤10 mtorr. Samples were dried overnight, and 200 mg of dried sample was added to 20 mL of heptane (at 4°C) in a cutoff 50-mL centrifuge tube housed in a 250-mL beaker containing partially frozen liquid heptane. The sample was sonicated for 30 s (power setting 3, 30% duty cycle), and filtered successively through 200- and 80-μm nylon nets (Tetko Inc., Briarcliff Manor, NY). The sample was precipitated by centrifugation at 100*g* for 3 min. Leaf pellets were resuspended in 1 mL of heptane (at 4°C), and each sample was loaded onto a 7-mL discontinuous gradient of heptane/tetrachloroethylene, which ranged from 1.35 to 1.60 g mL^{-1}. Samples were centrifuged at 4°C in a swinging bucket rotor (model HB-4, Sorvall Instruments, Newtown, CT) at 25,000*g* for 3 h. Gradients were fractionated into six portions from the top using a transfer pipette (typically about 1 mL for each). For marker assays, 0.5 mL was diluted with cold heptane in microfuge tubes, and the gradient material was precipitated by centrifugation at 10,000*g* for 5 min. The remaining gradient material from each fraction was diluted with heptane and collected by centrifugation. Residual heptane was evaporated overnight at 4°C under reduced pressure in a vacuum jar with desiccant and paraffin.

Marker Assays

The dried fractions were resuspended in 5 mL of buffer containing 0.1 M Bicine (pH 7.8), 5 mM $MgCl_2$, 1 mM EDTA, and 5 mM dithiothreitol. The samples were sonicated for 20 s (power setting 3, 30% duty cycle), and centrifuged at 10,000*g* for 5 min. Supernatants were used for enzyme assays and the pellet was used for Chl determinations. Chl was used for the chloroplast marker. Chl was determined by adding 1 mL of 80% (v/v) acetone to the pellet, sonicating for 30 s, centrifuging for 2 min in a microcentrifuge, and repeating the extraction twice. The concentration of Chl (a + b) was determined using the extinction coefficients of Porra et al. (1989).

Phospho*enol*pyruvate (PEP)-carboxylase (PEPC; E.C. 4.1.1.31) activity was used as a marker for the cytosol. PEPC activity was assayed as described by Krall and Edwards (1993), with 0.3 mM NADH, 5 units mL^{-1} malate dehydrogenase, and 2.5 mM PEP in a final volume of 1 mL. After a 5-min pre-incubation at 25°C, the reaction was initiated with PEP, and PEPC activity was measured as the reduction in NADH detected as a spectrophotometric decrease in A_{340}.

For the vacuolar marker, we used α-mannosidase (E.C. 3.2.1.24) activity, assayed in duplicate for 60 min at 37°C as described by Sharkey and Vanderveer (1989). Total protein was determined by the method of Bradford (1976).

DMAPP in Gradient Fractions

Dried gradient fractions were resuspended in 0.5 mL of distilled water, rapidly transferred to a 4.8-mL glass vial, acidified with 8 M H_2SO_4, and assayed as described above. In an initial experiment, the recovery of DMAPP from fractionated material was determined. The recovery of DMAPP added to Chl-containing fractions was 91%.

Data Analysis

The distribution of DMAPP was calculated according to a two-compartment, graphical analysis as described by Gerhardt and Heldt (1984) and Sharkey and Vanderveer (1989). Because DMAPP is thought to be largely associated with the chloroplast and cytosol, we calculated (from the appropriate graphs) the relative distribution of DMAPP between these two compartments. Because this analysis assumes that the DMAPP in each fraction is only from the chloroplast stroma and cytosol, it is necessary to estimate the contribution of the vacuole to determine the error in this analysis. Assuming all of the DMAPP in the heaviest fraction was vacuolar (an overestimate) and applying the ratio of DMAPP to vacuolar marker to the top fractions, less than 9% of the DMAPP in the top fractions came from vacuolar contamination. Because the majority of DMAPP (>70%) in multiple gradients consistently partitioned into the two lightest fractions, and were predominantly associated with the Chl fractions, we feel it is justifiable to apply this two-compartment approach.

ACKNOWLEDGMENTS

We are grateful for the efforts of Peter Vanderveer and Tom Sharkey in teaching Todd N. Rosenstiel the nonaqueous fractionation method.

Received January 22, 2002; accepted March 6, 2002.

LITERATURE CITED

Bach TJ, Boronat A, Campos N, Gerrer A, Vollack K-U (1999) Mevalonate biosynthesis in plants. Crit Rev Biochem Mol Biol **34:** 107–122

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Chameides WL, Lindasy RW, Richardson J, Kiang CS (1988) The role of biogenic hydrocarbons in urban photochemical smog: Atlanta as a case study. Science **241:** 1473–1475

Davisson VJ, Woodside AB, Poulter CD (1985) Synthesis of allylic and homoallylic isoprenoid pyrophosphates. Methods Enzymol **110:** 130–144

Edmond J, Popjak G (1974) Transfer of carbon atoms from mevalonate to *n*-fatty acids. J Biol Chem **249:** 66–71

Estévez JM, Cantero A, Reindl A, Reichler S, Léon P (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J Biol Chem **276:** 22901–22909

Fall R, Wildermuth MC (1998) Isoprene synthase: from biochemical mechanism to emission algorithm. J Geophys Res **103:** 25599–25609

Farré EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L (2001) Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiol **127:** 685–700

Fisher AJ (2001) Biosynthesis of C_5 oxygenated volatile organic compounds in plants. PhD thesis. University of Colorado, Boulder

Fisher AJ, Baker BM, Greenberg JP, Fall R (2000) Enzymatic synthesis of methylbutenol from dimethylallyl diphosphate in needles of *Pinus sabiniana*. Arch Biochem Biophys **383:** 128–134

Fisher AJ, Rosenstiel TN, Shirk MC, Fall R (2001) Nonradioactive assay for cellular dimethylallyl diphosphate. Anal Biochem **292:** 272–279

Gerhardt R, Heldt HW (1984) Measurement of subcellular metabolite levels in leaves by fractionation of freeze-stopped material in nonaqueous media. Plant Physiol **75:** 542–547

Graedel TE (1979) Terpenoids in the atmosphere. Rev Geophys Res Space Phys **17:** 937–947

Guenther A, Hewitt CN, Erickson D, Fall R, Geron C, Graedel T, Harley P, Klinger L, Lerdau M, McKay WA et al. (1995) A global model of natural volatile organic compound emissions. J Geophys Res **100:** 8873–8892

Harley P, Fridd-Stroud V, Greenberg J, Guenther A, Vasconcellos P (1998) Emission of 2-methyl-3-buten-2-ol by pines: a potentially large natural source of reactive carbon to the atmosphere. J Geophys Res **103:** 25479–25486

Harley PC, Monson RK, Lerdau MT (1999) Ecological and evolutionary aspects of isoprene emission by plants. Oecologia **118:** 109–123

Havel C, Rector ER II, Watson JA (1986) Isoprenoid synthesis in isolated embryonic *Drosophila* cells: possible regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity by shunted mevalonate carbon. J Biol Chem **261:** 10150–10156

Heintze A, Golrach J, Leuschner C, Hoppe P, Hagelstein P, Schylze-Siebert D, Schultz G (1990) Plastidic isoprenoid synthesis during chloroplast development. Plant Physiol **93:** 1121–1127

Hewitt CN, Kok GL, Fall R (1990) Hydroperoxides in plants exposed to ozone mediate air pollution damage to alkene emitters. Nature **344:** 56–68

Kesselmeier J, Staudt M (1999) Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. J Atmos Chem **33:** 23–88

Krall JP, Edwards GE (1993) PEP carboxylases from two C_4 species of *Panicum* with markedly different susceptibilities to cold inactivation. Plant Cell Physiol **34:** 1–11

Kreuzwieser L, Schnitzler JP, Sreinbrecher R (1999) Biosynthesis of organic compounds emitted by plants. Plant Biol **1:** 149–159

Kuzma J, Fall R (1993) Leaf isoprene emission rate is dependent on leaf development and the level of isoprene synthase. Plant Physiol **101:** 435–440

Lehning A, Zimmer I, Steinbrecher R, Bruggemann N, Schnitzler JP (1999) Isoprene synthase activity and its relation to isoprene emission in *Quercus robur* L. leaves. Plant Cell Environ **22:** 495–504

Leidreiter K, Kruse A, Heineke D, Robinson DG, Heldt HW (1995) Subcellular volumes and metabolite concentrations in potato (*Solanum tuberosum* cv. Désirée) leaves. Bot Acta **108:** 439–444

Lerdau M, Guenther A, Monson RK (1997) Plant production and emission of volatile organic compounds. BioScience **47:** 373–383

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol **50:** 47–65

Lichtenthaler HK, Rohmer M, Schwender J (1997) Two independent pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol Plant **101:** 643–652

Logan BA, Anchodoquy TJ, Monson RK, Pan R (1999) The effect of isoprene on the properties of spinach thylakoids and phosphatidylcholine liposomes. Plant Biol **1:** 602–606

Logan BA, Monson RK (1999) Thermotolerance of leaf discs from four isoprene-emitting species is not enhanced by exposure to exogenous isoprene. Plant Physiol **120:** 821–825

Logan BA, Monson RK, Potosnak MJ (2000) Biochemistry and physiology of foliar isoprene production. Trends Plant Sci **5:** 477–481

Loreto F, Mannozzi M, Maris C, Nascetti, Ferranti F, Pasqualini S (2001) Ozone quenching properties of isoprene and its antioxidant role in leaves. Plant Physiol **126:** 993–1000

Loreto F, Sharkey TD (1990) A gas exchange study of photosynthesis and isoprene emission in *Quercus rubra* L. Planta **182:** 523–531

Mahmoud SS, Croteau RB (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc Natl Acad Sci USA **98:** 8915–8920

Marinier E, Lincoln BC, Garneau M, David F, Brunengraber H (1987) Contribution of the shunt pathway of mevalonate metabolism to the regulation of cholesterol synthesis in rat liver. J Biol Chem **262:** 16936–16940

McCaskill D, Croteau R (1995) Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha* × *piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. Planta **197:** 49–56

Monson RK, Fall R (1989) Isoprene emissions from aspen leaves. Influence of environment and relation to photosynthesis and photorespiration. Plant Physiol **90:** 267–274

Monson RK, Harley PC, Litvak ME, Wildermuth M, Guenther AB, Zimmermann PR, Fall R (1994) Environmental and developmental controls over the seasonal pattern of isoprene emission from aspen leaves. Oecologia **9:** 260–270

Monson RK, Jaeger CH, Adams WW III, Driggers EM, Silver GM, Fall R (1992) Relationships among isoprene emission rate, photosynthesis, and isoprene synthase activity as influenced by temperature. Plant Physiol **98:** 1175–1180

Monson RK, Holland EA (2001) Biospheric trace gas fluxes and their control over tropospheric chemistry. Ann Rev Ecol Syst **32:** 547–576

Moore BD, Sharkey TD, Seeman JR (1995) Intracellular localization of CA1P and CA1P phosphatase activity in leaves of *Phaseolus vulgaris* L. Photosynth Res **45:** 219–224

Nes WD, Bach TJ (1985) Evidence for a mevalonate shunt in a tracheophyte. Proc R Soc Lond B **225:** 425–444

Niinemets Ü, Tenhunen JD, Harley PC, Steinbrecher R (1999) A model of isoprene emission based on energetic requirements for isoprene synthesis and leaf photosynthetic properties for *Liquidambar* and *Quercus*. Plant Cell Environ **22:** 1319–1335

Niyogi KK (2000) Safety valves for photosynthesis. Curr Opin Plant Biol **3:** 455–460

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta **975:** 384–394

Riens B, Lohaus G, Heineke D, Heldt HW (1991) Amino acid and sucrose content determined in the cytosolic, chloroplastic, and vacuolar compartments and in the phloem sap of spinach leaves. Plant Physiol **97:** 227–233

Sauer F, Schafer C, Neeb P, Horie O, Moortgat GK (1999) Formation of hydrogen peroxide in the ozonolysis of isoprene and simple alkenes under humid conditions. Atmos Env **33:** 229–241

Schnitzler J-P, Arenz R, Steinbrecher R, Lehning A (1996) Characterization of an isoprene synthase from leaves of *Quercus petraea* (Mattuschka) Liebl. Bot Acta **109:** 216–221

Schnitzler J-P, Lehning A, Steinbrecher R (1997) Seasonal pattern of isoprene synthase activity in *Quercus robur* leaves and its significance for modeling isoprene emission rates. Bot Acta **110:** 240–243

Sharkey TD, Loreto F (1993) Water stress, temperature, and light effects on the capacity for isoprene emission and photosynthesis of kudzu leaves. Oecologia **95:** 328–333

Sharkey TD, Vanderveer PJ (1989) Stromal phosphate concentration is low during feedback limited photosynthesis. Plant Physiol **91:** 679–684

Sharkey TD, Yeh S (2001) Isoprene emission from plants. Annu Rev Plant Physiol Plant Mol Biol **52:** 407–436

Silver GM, Fall R (1995) Characterization of aspen isoprene synthase, an enzyme responsible for leaf isoprene emission to the atmosphere. J Biol Chem **270:** 13010–13016

Stitt M, Lilley RMcC, Gerhardt R, Heldt HW (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. Methods Enzymol **174:** 518–522

Trainer M, Williams EJ, Parrish DD, Buhr MP, Allwine EJ et al. (1987) Models and observations of the impact of natural hydrocarbons on rural ozone. Nature **329:** 705–707

Wildermuth MC, Fall R (1996) Light-dependent isoprene emission. Characterization of a thylakoid-bound isoprene synthase in *Salix discolor* chloroplasts. Plant Physiol **112:** 171–182

Winter H, Robinson DG, Heldt HW (1993) Subcellular volumes and metabolite concentrations in barley leaves. Planta **191:** 180–190

Winter H, Robinson DG, Heldt HW (1994) Subcellular volumes and metabolite concentrations in spinach leaves. Planta **193:** 530–535

Zeidler J, Lichtenthaler HK (2001) Biosynthesis of 2-methyl-3-buten-2-ol emitted from needles of *Pinus ponderosa* via the non-mevalonate DOXP/MEP pathway of isoprenoid formation. Planta **213:** 323–326

Zimmer W, Bruggemann N, Emeis S, Giersch C, Lehning A, Steinbrecher R, Schnitzler J-P (2000) Process-based modeling of isoprene emission by oak leaves. Plant Cell Environ **23:** 585–595