

Leaf Isoprene Emission Rate Is Dependent on Leaf Development and the Level of Isoprene Synthase¹

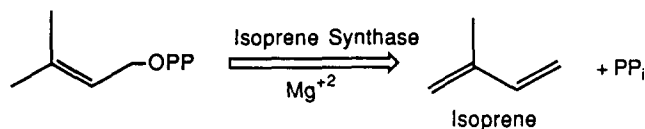
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Isoprene (2-methyl-1,3-butadiene) is a major volatile hydrocarbon produced by many plant species. Here we report that in velvet bean (*Mucuna* sp.), isoprene emission is strongly dependent on leaf developmental state and that changes in extractable isoprene synthase activity parallel isoprene emission rates during leaf development. Both leaf emission and enzyme activity exhibit over 100-fold increases from leaf emergence to leaf age 14 d and exhibit similar patterns to 23 d. This suggests that the enzyme, isoprene synthase, is responsible for the in vivo production of isoprene and that the level of the enzyme regulates the pattern of isoprene emission in response to leaf development.

Isoprene (2-methyl-1,3-butadiene) is emitted to the atmosphere by numerous plant species. Because this reactive hydrocarbon has important effects on the oxidation potential of the troposphere, much effort is underway to understand and model the production of isoprene from plant canopies (Trainer et al., 1987; Chameides et al., 1988; Jacob and Wofsy, 1988; Monson et al., 1991a). This modeling would be enhanced by an understanding of the biochemical, physiological, and phenological processes that regulate isoprene biosynthesis and emission from leaves (Guenther et al., 1991; Monson et al., 1991a, Fall and Monson, 1992).

Although isoprene emission from plants was discovered over 30 years ago (Sanadze, 1957), until now little was known about the biochemistry of isoprene formation. Recently, we have described an enzyme, termed isoprene synthase, in aspen leaf extracts (Silver and Fall, 1991). This enzyme catalyzes the conversion of DMAPP to isoprene:



This discovery has provided a new approach to studying the biochemical regulation and mechanism of isoprene production. For example, it has been known for years that isoprene emission rate is strongly dependent on leaf temperature and PAR (Sanadze and Kalandadze, 1966; Monson and Fall, 1989; Monson et al., 1991b; Sanadze, 1991; Sharkey et al., 1991; Monson et al., 1992). We have shown that the temperature dependence of aspen isoprene emission rate in

vivo parallels the temperature dependence of isoprene synthase activity in vitro, suggesting that regulation of leaf isoprene emission is accomplished primarily through the in vivo activity of the enzyme (Monson et al., 1992). The light dependence of isoprene emission rate may also be a reflection of the in vivo activity of the enzyme, perhaps by regulation of the flow of precursors into the mevalonate pathway (Loomis and Croteau, 1973; Gray, 1987), or by direct light/dark modulation of the enzyme (Anderson, 1986). The sub-cellular location of isoprene synthase activity has been difficult to establish, although a chloroplast location is consistent with published information (Mgaloblishvili, 1978; Sanadze, 1990).

Another important factor that influences isoprene emission rate is leaf development. We have reported that the isoprene emission rate for velvet bean (*Mucuna* sp.) increased as much as 125-fold as leaves developed, and then declined in older leaves (Grinspoon et al., 1991). In addition, in these leaves, photosynthetic competence developed several days before significant isoprene emission occurred.

Given the developmental dependence of leaf isoprene emission and the recent discovery of isoprene synthase, we set out to determine if whole leaf isoprene emission correlates with changes in the activity level of isoprene synthase. Isoprene emission of whole velvet bean leaves was compared with the extractable isoprene synthase activity during the course of velvet bean development. We also report some of the properties of velvet bean isoprene synthase.

MATERIALS AND METHODS

Reagents

DMAPP was synthesized and purified as previously described (Davisson et al., 1985). Reagents for the synthesis of DMAPP were supplied by Aldrich (Milwaukee, WI). The structure of DMAPP was confirmed by TLC and ¹H and ³¹P NMR using a 300-MHz Varian VXR-300S instrument. Polyclar AT was supplied by Serva Biochemicals (Westbury, NY). DEAE-Sephacel was obtained from Pharmacia (Piscataway, NJ). All other chemicals were reagent grade.

Plant Growth

Velvet bean (*Mucuna* sp.) seeds (Glendale Enterprises, DeFuniak Springs, FL) were grown in 5-L plastic pots in Metro Mix 350 (American Clay, Denver, CO) and fertilized

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weekly with Peters Professional Soluble Plant Food (Peters Fertilizer Products, Fogelsville, PA). Plants were grown in a greenhouse with supplemental lighting ($500 \mu\text{mol m}^{-2} \text{s}^{-1}$) of low pressure sodium vapor lamps (General Electric, Cleveland, OH) for an 18-h photoperiod. Temperatures ranged from 21°C (night) to 27°C (day). The velvet bean vines were supported on horizontal poles to maintain all leaves at constant light intensity. Leaf age was monitored by tagging leaves on the day they unfolded.

Leaf Isoprene Emission and CO₂ Assimilation Rates

Isoprene emission from velvet bean leaves was determined by taking leaf discs (1 cm²) from leaves of known age and incubating them in sealed vials under light as described previously (Grinspoon et al., 1991). Two milliliters of head space gas were taken after 20 min of incubation and analyzed for isoprene by GC (0.5-mL sample loop) as previously described (Silver and Fall, 1991). For each experiment, three leaves of the same age were taken from three different plants, and the isoprene emission rates were averaged. Chl determinations for the leaf discs were performed according to Arnon (1949). The same leaves used for these disc assays were subsequently used for enzyme extracts (described below).

Partial Purification of Isoprene Synthase

Extracts of velvet bean leaves were prepared according to the method previously published for aspen leaves (Silver and Fall, 1991) with two modifications. One, PEB contained 10 mM DTT rather than 1 mM DTT, and, two, 1 g of leaves was taken for extraction (approximately three leaves) rather than 5 g. Leaves of the same age were taken from three different plants, used for the disc assay, and subsequently extracted. Extracts were fractionated with ammonium sulfate as previously described (Silver and Fall, 1991). Eighty percent of isoprene synthase activity was found to precipitate between 40 and 55% saturation with ammonium sulfate. For each extract, the ammonium sulfate precipitate was resuspended in 1/10 volume of PEB containing 10 mM DTT and stored at -70°C. Extracts prepared and stored in this manner proved to be stable for at least 3 months.

For further purification of isoprene synthase, a DEAE-Sephacel chromatography step was used. Ammonium sulfate precipitates from different-aged leaves were dialyzed against PEB containing 10 mM DTT. A DEAE-Sephacel column (7-mL bed volume) was also equilibrated with this same buffer. After dialysis, the sample was applied to the column and eluted with a 0 to 0.5 M NaCl linear gradient in PEB. Fractions were tested for isoprene synthase activity (see enzymic assay) and protein concentration was determined by the Bradford assay (Bradford, 1976). Active fractions were concentrated as previously described (Silver and Fall, 1991) to approximately 2 mg of protein mL⁻¹. The concentrated samples were stored at -70°C and used for characterization of isoprene synthase activity.

Assay for Enzymic Isoprene Production

For the developmental enzymic assays, 10 μL of the 40 to 55% ammonium sulfate precipitates from different-aged leaves (10–20 μg of protein) were incubated with 10 mM DMAPP (10 μL of a 20-mM solution) at 30°C in 4-mL glass vials sealed with Teflon-lined septa. After 10 min of incubation, 2 mL of headspace were analyzed for isoprene production by GC (Silver and Fall, 1991). DEAE-Sephacel column fractions were analyzed similarly.

For pH optimum assays, three buffers at 12 pH values were used: Mes, pH 5.4 to 8.2; 1,3-bis[tris(hydroxymethyl)methylamino]propane, pH 7.0 to 9.4; and 3-(cyclohexylamino)-1-propanesulfonic acid, pH 9.5 to 11.2. In a reaction volume of 20 μL , 10 μg of DEAE-purified protein were incubated with 10 mM DMAPP, 100 mM of the appropriate buffer, and 20 mM MgCl₂. Nonenzymic assays contained the above, but instead of protein, PEB buffer was added. Assays for isoprene production were performed as above.

For metal-ion dependence of isoprene synthase, DEAE-purified protein was dialyzed against PEB containing no Mg²⁺. Ten micrograms of the dialyzed protein was incubated with various concentrations of Mg²⁺, Mn²⁺, and Ca²⁺ and 10 mM DMAPP. Nonenzymic controls contained PEB buffer in place of protein. Assays for isoprene production from these samples were performed as above.

RESULTS

Relation of Leaf Isoprene Emission Rate to Isoprene Synthase Activity

The developmental dependence of leaf isoprene emission could be dependent on the activity of isoprene synthase, an enzyme that we have recently characterized from aspen leaves (Silver and Fall, 1991; Monson et al., 1992). To investigate this possibility, we analyzed velvet bean leaves of different ages for isoprene emission rate and extractable isoprene synthase activity. Isoprene emission rates for individual leaves were analyzed by leaf disc assays, and then each leaf was extracted and analyzed for isoprene synthase. Experiments established that optimal conditions for isoprene synthase assay in velvet bean extracts were similar to those for the aspen enzyme (Silver and Fall, 1991; see below).

In four separate experiments, three leaves of the same age were taken from three different plants and tested for leaf isoprene emission rate and isoprene synthase activity. Leaf ages of 2 d (rapidly expanding) to 30 d (nearly senescent) were used for the studies, and among the four experiments, all leaf ages were covered in this range. One representative experiment is shown (Fig. 1). In each of the four experiments, a similar pattern emerged between leaf emission and enzymic activity, although the absolute activity varied. Over 100-fold increases in emission rate and enzymic activity occurred from leaf emergence to 14 d with a subsequent decline to 18 d, followed by a rise to 23 d. The rise and fall pattern of developmental activity (in velvet bean, to 20 d) is common to many developmentally regulated enzymes (refs. in Šesták and Zima, 1985). Differences in whole leaf and enzymic activity emerged between 23- and 30-d-old leaves: leaf emission kept rising and enzymic activity fell in older leaves.

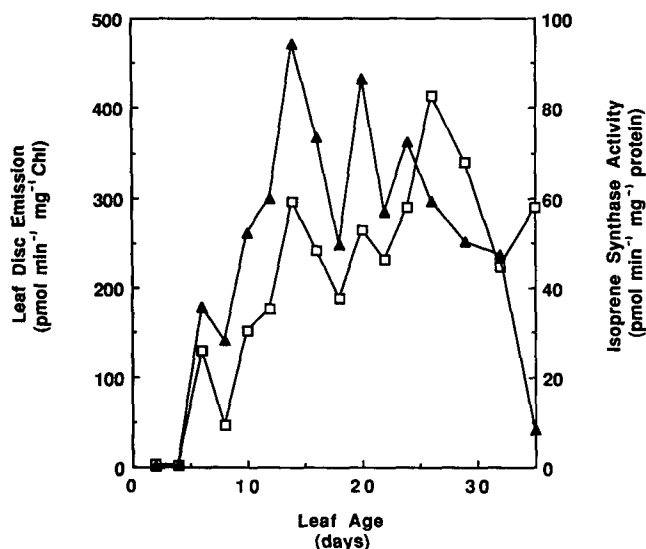


Figure 1. Comparison of leaf disc isoprene production to enzymic isoprene production during velvet bean leaf development. □, Leaf disc emission. ▲, Isoprene synthase activity. For leaf disc isoprene emission, discs were taken from three leaves of the same age and assayed for isoprene production as described in "Materials and Methods." The three samples were averaged for each age and plotted above. Emission rates are expressed on a per Chl basis. Enzymic rates are expressed on a per protein basis.

For each leaf age, normalized isoprene synthase activity was plotted against normalized leaf disc emission (Fig. 2). The normalization was necessary to compare four experiments on the same scale (i.e. absolute values for enzyme activity or leaf emission of a certain age differed among the four experiments, although the developmental pattern of these values remained constant). Normalization was performed by assigning the highest leaf emission or enzyme activity in a given experiment a value of 1. All lesser activities in that experiment were expressed as fractions of the highest activity. As a result, values in all four experiments ranged from 0 to 1, and thus could be compared. Linear regressions were applied to the normalized data, and correlation coefficients of 0.77 to 0.98 were exhibited, suggesting that extractable isoprene synthase activity is tightly linked to whole-leaf isoprene emission rates.

These findings suggest that changes in isoprene synthase activity during leaf development are regulated by long-term changes in the amount of enzyme or enzyme activity. However, one could also explain the results by the presence of an inhibitor of the enzyme in extracts with less activity (e.g. 0–14 d, 18–20 d, and over 26 d). This possibility was tested in two ways: first, by adding extracts with little to no activity to extracts with high activity to look for inhibition; and second, by partially purifying extracts from leaves of different ages to try to remove a putative inhibitor. In the addition experiments, no sign of inhibition by the less-active fractions was detected (data not shown). For the purification experiments, extracts from 4-, 6-, and 14-d-old leaves were partially purified by ammonium sulfate precipitation, followed by ion-exchange chromatography on DEAE-Sephacel. The amount

of active enzyme eluted from the DEAE-Sephacel column increased from extracts of 6- to 14-d-old leaves (Fig. 3), although the purification factor remained constant (data not shown), whereas no active enzyme was detected in comparable fractions from 4-d-old leaves (Fig. 3). In light of these results, the presence of a loosely bound isoprene synthase inhibitor seems unlikely.

Characterization of Velvet Bean Isoprene Synthase

This is the first report of isoprene synthase activity in a plant other than quaking aspen. Given this, we set out to determine if the isoprene synthases from aspen and velvet bean are similar. The DEAE-Sephacel elution profiles for the velvet bean enzyme and aspen enzyme are similar (Fig. 3 and figure 1 in Silver and Fall, 1991). In addition, the pH optimum for velvet bean isoprene synthase was approximately 7.8 to 8.5 (Fig. 4A), correlating strongly with the optimal pH for aspen enzyme catalysis (Silver and Fall, 1991). Figure 4A also shows that the nonenzymic rate of DMAPP conversion to isoprene is insignificant at physiological pH values and is catalyzed only by strong acidic or basic conditions.

The metal ion dependence of the velvet bean enzyme was tested with Mg^{2+} , Mn^{2+} , and Ca^{2+} . The effects of all three metal ions on the nonenzymic reaction were insignificant in relation to the effects of the metals on the enzymic reaction. Either Mg^{2+} or Mn^{2+} was necessary for the enzymic conversion of DMAPP to isoprene; with Mg^{2+} , the maximal catalysis rate was 20 times higher than with Mn^{2+} in the concentration range of 0 to 30 mM (data not shown). Ca^{2+} at low concentrations (0–2 mM) stimulates the enzymic reaction; however,

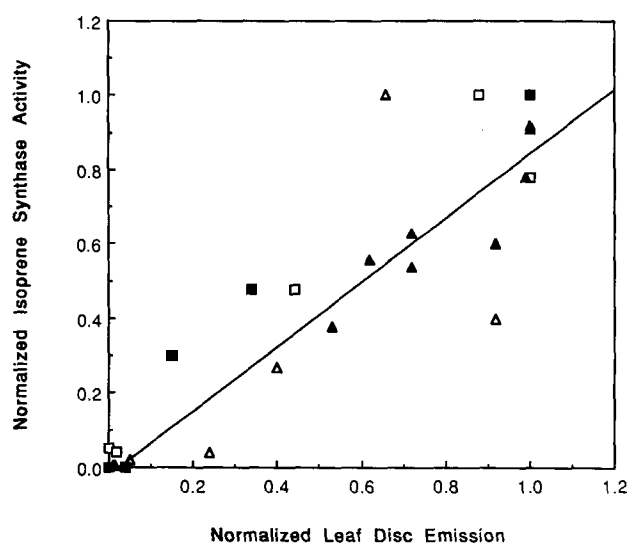


Figure 2. Correlation of velvet bean leaf disc isoprene production to isoprene synthase activity in leaves of different ages. Leaf disc isoprene emission rates and isoprene synthase activities from four experiments were normalized (scale 0–1) and plotted. Data for leaf ages up to 23 d were taken from experiments performed as in Figure 1. Four symbols (Δ , \blacktriangle , \square , \blacksquare) represent four different experiments. Linear regressions were performed and correlation coefficients were 0.77, 0.96, 0.96, and 0.98.

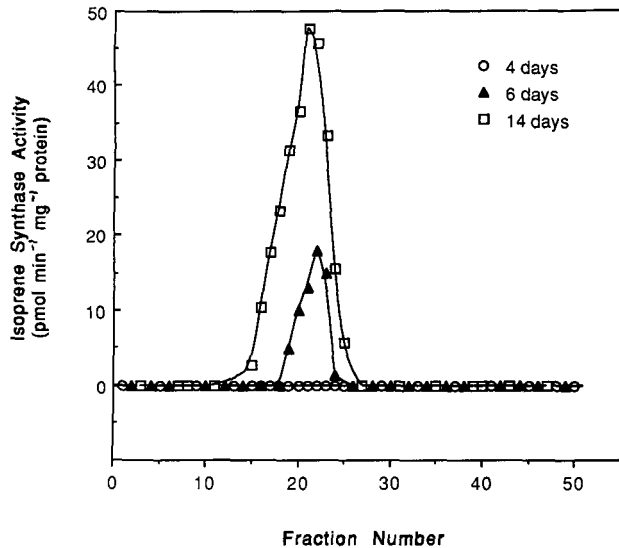


Figure 3. DEAE-Sephacel chromatography of leaf extracts from velvet bean leaves of three different ages. \circ , Extract from 4-d-old leaves exhibiting very little isoprene synthase activity. \blacktriangle , Extract from 6-d-old leaves exhibiting intermediate activity. \square , Extract from 14-d-old leaves exhibiting high activity. In each case, crude extracts were fractionated with ammonium sulfate (40–55% saturation) and then chromatographed on a DEAE-Sephacel column as described in "Materials and Methods." The enzyme was eluted with a linear 0 to 0.5 M NaCl gradient from fractions 1 to 50. Most protein eluted between fractions 15 and 20, to the left of the isoprene activity peak (data not shown). Protein and enzymic assays were performed as in "Materials and Methods."

high Ca^{2+} concentrations (2–30 mM) inhibit the enzymic reaction (data not shown). The maximal rate of catalysis was achieved at 15 mM Mg^{2+} (Fig. 4B). The Mg^{2+} dependence of the velvet bean enzyme is similar to that obtained with the aspen enzyme (Silver and Fall, 1991).

DISCUSSION

With the recent discovery of the enzyme isoprene synthase (Silver and Fall, 1991), we were able to test the idea that the developmental dependence of leaf isoprene emission is a function of the activity level of this enzyme. Here we report a dramatic increase in isoprene synthase activity during leaf development, with most of the increase occurring after leaves have fully expanded (8–12 d). Up to 23 d there is a strong correlation between leaf isoprene emission rate and the extractable isoprene synthase activity of leaves. We suggest that these results stem from long-term regulation of the level of isoprene synthase activity. Such regulation could occur as a result of a change in the amount of enzyme or irreversible covalent modification of the enzyme (e.g. posttranslation processing) (Gray, 1987). Future experiments with specific antibodies to isoprene synthase should reveal whether developmental changes in leaf isoprene emission result from changes in the amount of enzyme protein.

Short-term changes in leaf isoprene synthase activity are also likely. For example, isoprene emission rate decreases

rapidly (i.e. in minutes) after light-to-dark transitions (Monson et al., 1991b). This could result from short-term changes in the levels of metabolites or reversible covalent modification of the enzyme. We cannot currently explain the divergence between leaf and enzymic activity beyond 23 d; perhaps short-term regulation plays a larger role during this stage. For example, an increase in substrate (i.e. DMAPP) concentration due to changes in leaf metabolism with age could result in increased isoprene production despite decreased activity of isoprene synthase.

Many enzymes, such as Rubisco isolated from *Pisum sativum* L. and *Solanum tuberosum* L., exhibit a single rise/fall

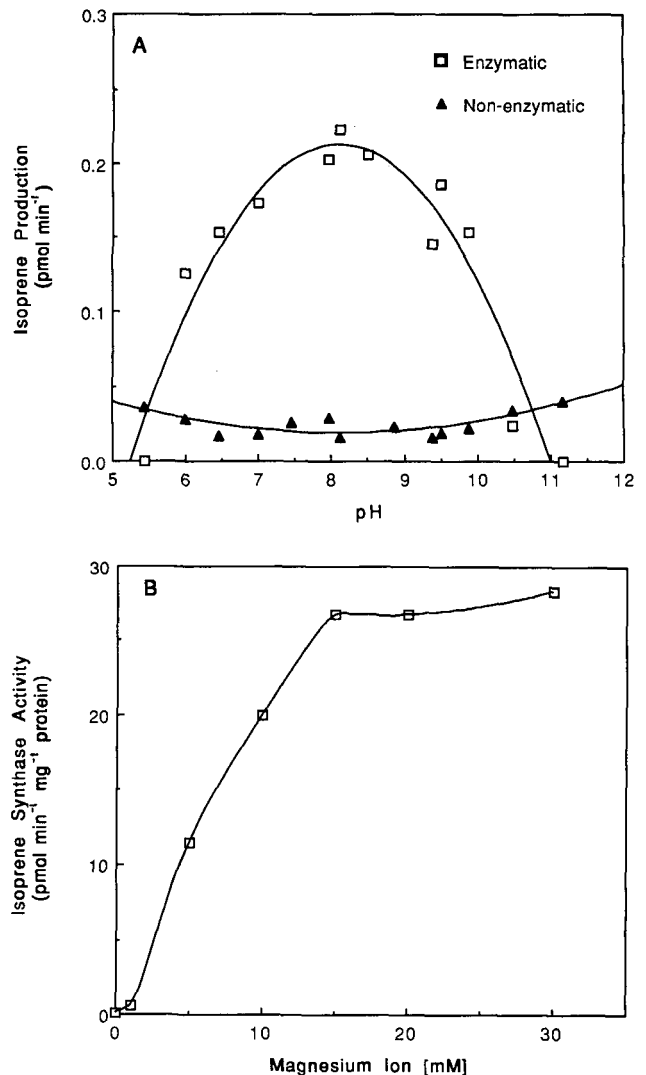


Figure 4. pH and Mg^{2+} dependence of velvet bean isoprene synthase. A, pH dependence. DEAE-Sephacel-purified protein was assayed for isoprene production at 12 pH values. The nonenzymic rate of isoprene production from DMAPP was also assayed at each pH. One of two representative experiments is shown. B, Mg^{2+} dependence. DEAE-Sephacel-purified protein was assayed for isoprene production at various concentrations of Mg^{2+} ; nonenzymic rates were subtracted in each case. One of two representative experiments is shown.

pattern similar to isoprene synthase from 0 to 20 d (refs. in Šesták and Zima, 1985). Isoprene synthase diverges from this pattern in late leaf development (20–30 d), exhibiting a second rise and fall. Although this is not typical for most developmentally controlled enzymes, some examples of divergence from the common pattern do exist in the literature, namely ribose-5-phosphate isomerase in *Pisum sativum* L. and Rubisco in *Phaseolus vulgaris* (French bean) (Gordon et al., 1978; Zima et al., 1981). In early stages, most enzymic developmental patterns can be attributed to the development of the leaf's photosynthetic capabilities (i.e. chloroplast development), hence the initial rise in activity pattern. The subsequent breakdown of these capabilities during senescence may contribute to the decrease in enzymic activity. We are currently unable to explain the complex pattern that we see with velvet bean isoprene synthase; however, because of its emergence in numerous replications of the experiment, we believe that the pattern is not an artifact.

The optimal pH for velvet bean isoprene synthase is approximately 7.8 to 8.5. Isoprene production is strongly influenced by light levels, and during the light reactions of photosynthesis, the pH of the stroma reaches 8.0. It is possible that isoprene synthase is present in the stroma and its activity is enhanced by a change in pH upon incubation in the light. We are currently exploring the subcellular location of isoprene synthase in the cell. It was previously suggested that the link of isoprene production to light was nonenzymic: if DMAPP is present in the thylakoid lumen, it could be converted to isoprene due to the acidification of the thylakoids during the light reactions of photosynthesis (Sanadze, 1990). This does not seem likely given the low nonenzymic rates of isoprene production when compared with the enzymic rates. When leaf isoprene emission rates, isoprene synthase activity, and the nonenzymic production of isoprene are all expressed on a per gram basis for a given age (e.g. 10-d sample in Fig. 1), the enzymic rate ($163 \text{ pmol min}^{-1} \text{ g}^{-1} \text{ leaf}$) can account for approximately 80% of whole leaf emission ($213 \text{ pmol min}^{-1} \text{ g}^{-1} \text{ leaf}$), whereas the nonenzymic rate ($7.4 \text{ pmol min}^{-1} \text{ g}^{-1} \text{ leaf}$) can account for less than 4%.

Perhaps the most significant aspect of this work is that it provides evidence that isoprene synthase is largely responsible for the *in vivo* production of isoprene in whole leaves. Silver and Fall (1991) first raised this possibility. Subsequently, when temperature curves for leaf isoprene emission and for enzymic catalysis were compared, they were virtually identical, suggesting that leaf isoprene emission is governed by the activity of the enzyme (Monson et al., 1992). In this developmental study, we have come to the same conclusion. Understanding the regulation of isoprene synthase activity may provide new approaches to modeling isoprene emission from forest canopies. Models may incorporate biochemical information for isoprene production rates. This biochemical information should reflect the steady-state rate of isoprene emission (Monson et al., 1991a).

Studies such as these will shed light on many unanswered questions concerning the regulation of isoprene emission from plants. It remains a mystery why some plant species produce isoprene and others do not. In the future, we hope to address this question by investigating the genetic and

enzymic similarities and differences between isoprene emitters and nonemitters.

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