Title of the thesis

On the physiology and evolution of volatile isoprenoid emission in plants

Name of the PhD candidate

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Educational qualification of the candidate

Master of Research (University of Bristol, UK)

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Declaration:

I certify that the work in this thesis entitled "*On the physiology and evolution of isoprenoid emission in plants*" has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

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***Thesis abstract*:**

Isoprenoid emission by plants (1 PgC/yr) via the chloroplast localised methyl-erythritol phosphate (MEP) pathway is a significant source of carbon that influences atmospheric oxidation chemistry and global carbon cycle. Despite significant progress in isoprenoid research, many deeper questions surround the biochemical regulation of the MEP pathway and the physiological adaptations of emitters that determine the observed diversity in emission rates, hindering our ability to estimate and forecast global emissions. In this thesis, we present the results of a comprehensive set of experiments on the physiology of isoprenoid emission in selected species of *Eucalyptus*, a diverse native Australian tree genus (>900 species) that is adapted to a wide ranging climate and habitats in Australia and emits both isoprene and monoterpenes at significant measurable quantities. Addressing some of the outstanding questions in complex interactive effects of abiotic stresses on isoprenoid emission, our experiments show how increased isoprenoid emission rate by plants under abiotic stresses (low CO_2, heat and drought) is sustained by increased ratio of electron transport rate to net carbon assimilation rate. We also show how the MEP pathway competes with photorespiration for the residual reducing power not invested in carbon assimilation. Our results highlight the importance of species-specific tolerance for drought and provide a basis to observed variability and uncertainty in emission responses to drought. Tackling another important question pertaining annual cycles in emission, we establish an independent role for photoperiod in seasonal oscillations in emission. Experiments show that temperature entrains seasonal rhythms in isoprenoid emission rates and it is likely gated by a photoperiodic clock. Sensitivity of emission to photoperiod means that increasing global mean temperature could interfere with the photoperiod signaling pathway in major emitting tree genera, altering their seasonal emission responses. Using a comprehensive data set, we trace the patterns in the origin and evolution of isoprene emission in land plants and propose a novel hypothesis. We discuss various levels of natural selection acting on isoprenoid emission whilst we put the most important of our experimental results in a global perspective.

Thesis abstract in Kannada (mother tongue of the PhD candidate)

ಸಸ್ಯಗಳಲ್ಲಿರುವ ಹರಿತ್ಕೋಶ(chloroplast)ಗಳಲ್ಲಿ ದ್ಯುತಿಸಂಶ್ಲೇಷಣ ಕ್ರಿಯೆ (photosynthesis) ನಡೆಯುತ್ತದೆ. ಅದೇ ಹರಿತ್ಕೋಶಗಳಲ್ಲಿ ನಡೆಯುವ **ಮೀಥೈಲ್ ಎರಿಥ್ರಿಟಾಲ್ ಫಾಸ್ಫೇಟ್ (MEP) ಪಾಥ್ವೇ** ಎಂಬ ಹಲವು ಹಂತಗಳ ಜೀವ ರಾಸಾಯನಿಕ ಪ್ರಕ್ರಿಯೆಗಳ ಮೂಲಕ ಐಸೋಪ್ರೀನೋಯ್ಡ್-ಗಳೆಂಬ ಅನಿಲರೂಪದ ಹೈಡ್ರೋಕಾರ್ಬನ್ ಗಳನ್ನೂ ಸಸ್ಯಗಳು ಉತ್ಪಾದಿಸಿ, ಜಗತ್ತಿನಾದ್ಯಂತ ವರ್ಷಕ್ಕೆ ಸುಮಾರು ಒಂದು ನೂರು ಕೋಟಿ ಟನ್ನು (1 Peta gram) ಗಳಷ್ಟು ಪ್ರಮಾಣದ ಇಂಗಾಲ (ಕಾರ್ಬನ್) ಅನ್ನು ವಾತಾವರಣಕ್ಕೆ ಹೊರ ಸೂಸುತ್ತವೆ. ಈ ಹೈಡ್ರೋಕಾರ್ಬನ್ ಗಳು ಭೂಮಿಯ ವಾತಾವರಣದ ಮೇಲೆ ಅನೇಕ ಪ್ರಭಾವಗಳನ್ನು ಬೀರುತ್ತವೆ. ಅವುಗಳಲ್ಲಿ (ಅ) ಭೂಮಿಯ ಮೇಲ್ಮೈಗೆ ಹತ್ತಿರವಾದ ವಾತಾವರಣದ (ಟ್ರೋಪೋಸ್ಫಿಯರ್) ಹೊರ ಪದರದಲ್ಲಿ (ಟ್ರೋಪೋಸ್ಫಿಯರ್) ಮನುಷ್ಯನಿರ್ಮಿತ ಯಂತ್ರಜನ್ಯ ನೈಟ್ರಿಕ್ ಆಕ್ಸೈಡ್-ಗಳೊಂದಿಗೆ ಸೇರಿಕೊಂಡು ಓಜೋನ್ (O_3) ಎಂಬ ವಿಷಕಾರಿ ಅನಿಲದ ಉತ್ಪತ್ತಿಗೆ ಕಾರಣವಾಗುವುದು (ಆ) ಮಳೆಮೋಡ ಹುಟ್ಟಿಕೊಂಡು ಬೆಳೆಯಲು ಸಹಾಯ ಮಾಡುವ ಮಳೆ ಬೀಜಗಳಾದ ಸೆಕೆಂಡರಿ ಏರೋಸೋಲ್-ಗಳ ಉತ್ಪತ್ತಿಗೆ ಸಹಾಯ ಮಾಡುವುದು ಮತ್ತು (ಇ) ಇಂಗಾಲದ ಭೂ-ಜೀವ ರಾಸಾಯನಿಕ ಚಕ್ರವನ್ನೂ (carbon cycle) ಪ್ರಭಾವಿಸುವುದು ಸಸ್ಯಜನ್ಯ ಐಸೋಪ್ರೀನೋಯ್ಡ್-ಗಳ ಮುಖ್ಯ ಪರಿಣಾಮಗಳು. ಜೊತೆಗೆ, ಕೆಲವು ಐಸೋಪ್ರೀನೋಯ್ಡ್-ಗಳು ಸಸ್ಯಗಳ ಪರಾಗ ಸ್ಪರ್ಶಕ್ಕೆ ಸಹಾಯಮಾಡುವ ಕೀಟಗಳನ್ನು ಆಕರ್ಷಿಸುವಲ್ಲಿ, ಸಸ್ಯಗಳನ್ನು ತಿನ್ನುವ ಕೀಟ, ಜಂತುಗಳನ್ನು ನಿಗ್ರಹಿಸುವಲ್ಲಿಯೂ ಕಾರ್ಯ ನಿರ್ವಹಿಸುತ್ತವೆ. ಹಲವು ದಶಕಗಳ ನಿರಂತರ ಸಂಶೋಧನೆಯ ಬಳಿಕವೂ, MEP ಪಾಥ್ವೇಯ ಕಾರ್ಯ ವಿವರಗಳ ಪರಿಚಯ ಮಾಡಿಕೊಂಡ ಮೇಲೂ, ಸಸ್ಯಗಳು ಈ ಐಸೋಪ್ರೀನೋಯ್ಡ್-ಗಳನ್ನು ಯಾವಾಗ, ಏಕೆ, ಎಷ್ಟು ಮಟ್ಟದಲ್ಲಿ ಹೊರಸೂಸುತ್ತವೆಂಬುದನ್ನು ನಾವು ಸಂಪೂರ್ಣವಾಗಿ ಅರ್ಥಮಾಡಿಕೊಳ್ಳಲು ಸಾಧ್ಯವಾಗಿಲ್ಲ. ಆಸ್ಟ್ರೇಲಿಯಾದಲ್ಲಿರುವ ನೀಲಗಿರಿ ಮರಗಳ ವೈವಿಧ್ಯತೆ (800 ಕ್ಕೂ ಹೆಚ್ಚು ಪ್ರಬೇಧಗಳು) ಮತ್ತು ಅವು ತೋರಿಸುವ ಪರಿಸರ ಸಂಬಂಧಿ ಸಸ್ಯಶರೀರ ಮಾರ್ಪಾಡುಗಳು ಅನ್ಯಾದೃಶ. ಅವುಗಳಲ್ಲಿ ಕೆಲವು ಉತ್ತಮ ಪ್ರತಿನಿಧಿ ಪ್ರಬೇಧಗಳನ್ನು ಉಪಯೋಗಿಸಿಕೊಂಡು, ಐಸೋಪ್ರೀನೋಯ್ಡ್ ಹೊರ ಸೂಸುವಿಕೆಯನ್ನು ಸಸ್ಯ ಶರೀರಶಾಸ್ತ್ರ ಹಿನ್ನೆಲೆಯಿಂದ ಪ್ರಯೋಗಕ್ಕೊಳಪಡಿಸಿ, ಕೆಲವು ಗಹನವಾದ ಪ್ರಶ್ನೆಗಳಿಗೆ ಉತ್ತರ ಹುಡುಕುವ ಪ್ರಯತ್ನವನ್ನು ಈ PhD ಮಹಾಪ್ರಬಂಧದಲ್ಲಿ ಮಾಡಲಾಗಿದೆ.

ಹರಿತ್ಕೋಶದಲ್ಲಿ ಸಂಭವಿಸುವ MEP ಪಾಥ್ವೇ ಯು ದ್ಯುತಿಸಂಶ್ಲೇಷಣ ಕ್ರಿಯೆಯಿಂದ ಉತ್ಪಾದಿಸಲ್ಪಡುವ ರಾಸಾಯನಿಕ ಶಕ್ತಿಯುಕ್ತ ATP ಮತ್ತು NADPH ಗಳನ್ನು ಅವಲಂಬಿಸಿರುತ್ತದೆ. ಆದರೆ MEP ಪಾಥ್ವೇನಂತೆಯೇ ಇತರ ಅನೇಕ ರಾಸಾಯನಿಕ ಪ್ರಕ್ರಿಯೆಗಳು ATP ಮತ್ತು NADPH ಗಳಿಗಾಗಿ ಸ್ಪರ್ಧಿಸುತ್ತವೆ. ಈ ತೆರನಾದ ಸೂರ್ಯಕಿರಣ ಜನ್ಯ ರಾಸಾಯನಿಕ ಶಕ್ತಿಗಾಗಿ MEP ಪಾಥ್ ವೇಯು ಸ್ಪರ್ಧಿಸುತ್ತದೆ ಮತ್ತು MEP ಪಾಥ್ ವೇಯ ಕಾರ್ಯ ಶೀಲತೆಯು ಹೆಚ್ಚಾಗುವುದಕ್ಕೆ ದ್ಯುತಿಸಂಶ್ಲೇಷಣೆಯ ಎರಡು ಭಾಗಗಳ (ಬೆಳಕು ಅವಲಂಬಿತ ಮತ್ತು ಬೆಳಕನ್ನವಲಂಬಿಸದ ಕ್ರಿಯೆಗಳ) ನಡುವೆ ಉಂಟಾಗುವ ಅಸಮತೋಲನವು ಕಾರಣ ಎಂದು ನಾವು ಪ್ರತಿಪಾದಿಸಿದ್ದೇವೆ. ಬರಗಾಲದ ಒತ್ತಡದಲ್ಲಿರುವಾಗ ಸಸ್ಯಗಳು ಹೇಗೆ ತಮ್ಮ ಐಸೋಪ್ರೀನೋಯ್ಡ್ ಹೊರಸೂಸುವಿಕೆಯನ್ನು ಹೆಚ್ಚಿಸುತ್ತವೆ ಎಂಬುದನ್ನು ನಮ್ಮ ಪ್ರಯೋಗಗಳು ಆಧಾರಸಹಿತವಾಗಿ ವಿಶದಪಡಿಸುತ್ತವೆ. ಹಾಗೆಯೇ, ವಾರ್ಷಿಕ ಋತುಚಕ್ರಗಳ ಪ್ರಭಾವದಿಂದ ಐಸೋಪ್ರೀನೋಯ್ಡ್ ಹೊರಸೂಸುವಿಕೆಯು ಬೇಸಿಗೆಯಲ್ಲಿ ಹೆಚ್ಚಾಗಿ, ಚಳಿಗಾಲದಲ್ಲಿ ಕಡಿಮೆಯಾಗುವುದು ವಿಜ್ಞಾನಿಗಳಿಗೆ ಗೊತ್ತಿದೆ. ಆದರೆ ಈ ಏರಿಳಿತಗಳುಂಟಾಗಲು ಋತುಸಹಜ ತಾಪಮಾನ ವೈಪರೀತ್ಯಗಳೇ ಸಾಕೇ ಎಂಬ ಪ್ರಶ್ನೆ ನಮ್ಮ ಮುಂದಿತ್ತು. ಸಸ್ಯಗಳು ಋತುಚಕ್ರಕ್ಕನುಗುಣವಾಗಿ ಸೂರ್ಯನ ಬೆಳಕಿನ ಕಾಲಪ್ರಮಾಣದಲ್ಲುಂಟಾಗುವ ಬದಲಾವಣೆಗಳನ್ನೂ ಗ್ರಹಿಸುವುದರ ಮೂಲಕ MEP ಪಾಥ್ವೇಯ ಜೀನ್ (ವಂಶವಾಹಿ)ಗಳನ್ನು ವ್ಯಕ್ತಪಡಿಸಿ, ತಮ್ಮ ಐಸೋಪ್ರೀನೋಯ್ಡ್ ಹೊರಸೂಸುವಿಕೆಯನ್ನು ನಿಯಂತ್ರಿಸುತ್ತವೆಂದು ನಮ್ಮ ಪ್ರಯೋಗ ಫಲಿತಾಂಶಗಳು ತೋರಿಸಿವೆ. ಮನುಷ್ಯನ ಚಟುವಟಿಕೆಗಳಿಂದ ಈಗ ಉಂಟಾಗುತ್ತಿರುವ ಭೂಮಿಯ ತಾಪಮಾನ ಏರಿಕೆಯಿಂದ ಮುಂದೆ ಸಸ್ಯಗಳ ಬೆಳಕು ಮತ್ತು ತಾಪಮಾನ ಗ್ರಹಿಸುವ ಸಂಕೀರ್ಣ ಪ್ರವಹನ ವ್ಯವಸ್ಥೆಯ ಮೇಲೆ ವ್ಯತಿರಿಕ್ತ ಪರಿಣಾಮ ಉಂಟಾಗಿ ಅವುಗಳ ಐಸೋಪ್ರೀನೋಯ್ಡ್ ಹೊರಸೂಸುವಿಕೆ ಮತ್ತು ಇತರ ಹರಿತ್ಕೋಶೀಯ ಕ್ರಿಯೆಗಳು ಬದಲಾವಣೆಗಳಿಗೆ ಒಳಗಾಗುವ ಸಾಧ್ಯತೆಗಳಿವೆ. ಇಷ್ಟೆಲ್ಲ ವಿಚಾರಗಳನ್ನು ಚರ್ಚಿಸಿದ ಮೇಲೆ, ಸಸ್ಯಜನ್ಯ ಐಸೋಪ್ರೀನೋಯ್ಡ್ ಹೊರಸೂಸುವಿಕೆಗೆ ಸಂಬಂಧಿಸಿದ ಎಲ್ಲ ಹೊಸ ಆಧಾರಗಳನ್ನು ಪರಾಮರ್ಶಿಸಿ, ಏಕೆ ಕೆಲವು ಸಸ್ಯಗಳು ಮಾತ್ರ ಐಸೊಪ್ರೀನ್ (ಐಸೊಪ್ರೀನೋಯ್ಡ್ ಗಳಲ್ಲಿ ಒಂದು ಬಗೆ) ಅನ್ನು ಉತ್ಪಾದಿಸುತ್ತವೆ? ಮತ್ತು ಏಕೆ ಬಹುತೇಕವು ಉತ್ಪಾದಿಸುವುದಿಲ್ಲ? ಎಂಬ ಪ್ರಶ್ನೆಗಳನ್ನು ಜೀವ-ವಿಕಾಸವಾದದ ದೃಷ್ಟಿಯಿಂದ ಜಿಜ್ಞಾಸೆಗೊಳಪಡಿಸಿದ್ದೇವೆ. ಹಾಗೆ ಮಾಡುವುದರ ಜೊತೆಗೆ, ಈ ವಿಸ್ಮಯಕಾರಿ ಪ್ರಕ್ರಿಯೆಯ ಪ್ರಾಮುಖ್ಯತೆಯನ್ನು ಇಂದಿನ ವೈಜ್ಞಾನಿಕ ಬೆಳವಣಿಗೆಗಳ ಮತ್ತು ಅಗತ್ಯಗಳ ಹಿನ್ನೆಲೆಯಲ್ಲಿ ನೋಡಿ, ಮುಂದಿನ ಹಾದಿಯತ್ತ ಇಣುಕುವ ಪ್ರಯತ್ನದೊಂದಿಗೆ ಪ್ರಬಂಧ ಮುಕ್ತಾಯವಾಗುತ್ತದೆ.

Musings of a *Doctor of Philosophy* to be…

It is not easy to look back at a journey that almost refused to begin despite several attempts. Often I wasn't even close to being good enough for opportunities to like me. Sometimes opportunities didn't like me although I probably was one among many equals. It was a classic story of 'so near yet so far' many times over. I am where I am today mostly because of my past failures and because I didn't fail them.

It took me many years to recognize that there are people in this world who will be on my side even when they have nothing to gain from me. Most of them were strangers in different countries who taught me the art of trust when my instincts either deserted me or had no use. Some of those strangers have become my friends today. I am indebted to their support and understanding.

I am sad to say that some people remained strangers even after several years of interaction and exchange of ideas. Even after trying hard, I couldn't understand many of them. Some interactions were forced on us by circumstances. I could blame myself for not allowing those relationships to grow and that would be true. I have to say with sadness that I couldn't be faulted in cases where people remained complete strangers to me apparently because of their own prejudices and complexes. Such interactions have continued only because the people involved (including me) wanted to preserve self-interest at all costs. I am not proud of them. Nonetheless, some of those interactions had a significant role to play in my journey and need to be acknowledged.

I love trees. I have dreamed about trees, about what they do and what they mean ever since I was 6 or 7 years old. If there is anything that I love equally, it is to interact with the elderly. I am what I am today because of my grandmothers and grandfathers (some related to me and many not so). My teachers from primary school onwards, professors in colleges and universities have shaped my thoughts. I have learnt about my past and seen my future through their eyes. As I speak, my father and my mother are as relieved as I am about the fact that a major milestone is in sight. They are happier than I am. Today, I am in a privileged position because of their sacrifices. A lot is still left for me to do. These are unspoken and unwritten promises that will be reflected upon by someone younger to me 50 to 100 years from now.

I feel as though I am standing at a precipice. The optimist in me feels the launching pad underneath my feet that would help me take a thrilling "leap of faith". The pragmatist in me feels relieved that the wait is almost over. The pessimist in me recognizes the precipice for what it really means…it would be foolish and costly if I lose sight of where I am.

September 18, 2014
K G Srikanta Dani

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I feel a deep sense of gratitude to A/Prof Brian Atwell, my PhD supervisor, for taking me on as a student even though he was as new to this field as I was when I began. He gave me freedom to plan my experiments and allowed me to grow as an independent researcher. I am equally grateful to Dr Ian Jamie, my associate supervisor for helping me design and assemble what turned out to be a complicated experimental set up. His reassuring presence had a calming influence on me and he made time for me whenever I needed him. I came to Macquarie University (MQ) because of Prof Colin Prentice (then at University of Bristol). He sowed the seeds of isoprene in my mind and nurtured it when it was young. After he and I both came to Macquarie University, I presented a road map for my PhD during a meeting with all three of my supervisors. After the meeting Prof Prentice wrote to me and I quote "*A great presentation this morning, and an exciting project! It's excellent that you're here (Sydney). In Brian Atwell's hands, you will be very well supervised... but of course I also look forward to seeing how this work develops, and helping where I can*". And many years later, we are here.

At Macquarie University, I met Prof Roger Hiller who became my unofficial supervisor in the lab. His no-nonsense approach improved clarity of my thoughts and experiments. I am grateful to him for being a mentor both within and outside the laboratory. I attended many western classical concert rehearsals of the Ku-ring-gai philharmonic orchestra (based in Sydney) with whom Prof Hiller is a long standing violist. Those were fine moments and I will cherish them.

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…and I acknowledge the support from my parents (S Latha and K R Ganesha), my sisters (Padmalekha and Geetha), my fiancée (Akhila), my relatives and friends in India.

I would like to dedicate this thesis to my paternal Aunt Mrs K Subbamma (1935-2014) who was my teacher and guardian in India.

September 22, 2014
K G Srikanta Dani

Citation for the publications emanating from this PhD thesis

1. **Dani, K.G.S.,** Jamie, I.M., Prentice, I.C., Atwell, B.J. **(2014)**, Increased ratio of electron transport to net assimilation rate supports elevated isoprenoid emission rate in eucalypts under drought, ***Plant Physiology,*** 166: 1059-1072
2. **Dani, K.G.S.,** Jamie, I.M., Prentice, I.C., Atwell, B.J. **(2014)**, Evolution of isoprene emission capacity in plants, ***Trends in Plant Science***, 19: 439-446
3. **Dani, K.G.S.,** Fineschi, S., Loreto, F. **(2015)**, Biogenic volatile isoprenoid emission and levels of natural selection. ***Journal of the Indian Institute of Science*** (Journal centenary issue), 95, 1-13
4. **Dani, K.G.S.,** Jamie, I.M., Prentice, I.C., Atwell, B.J **(2015)**, Species-specific photorespiratory rate, drought tolerance and isoprene emission rate in plants. ***Plant Signaling & Behavior,*** 10:3, e990830, 1-3
5. Harrison, S.P., Morfopoulos, C., **Dani, K.G.S.,** Prentice, I.C., Arneth, A., Atwell, B.J., Barkley. P., Leishman, M., Loreto, F., Medlyn, B., Niinemets, Ü., Peñuelas, J., Possell, M., Wright, I.J. **(2013)**, Volatile isoprenoid emission from plastids to planet, ***New Phytologist***, 197: 49-57

Contributions:

Contributions by individual authors are listed as a footnote at the beginning of each chapter.

Chapter 1

Introduction

Prelude

> *One would hardly have guessed that the delicious perfumes of acacia flowers or of orange blossoms had anything in common with the liver oils of the flounder, mackerel, or shark; that one of the products of the dry distillation of rubber was the chief building block used by nature in the construction of the growth-stimulating vitamin A, as well as of the green (chlorophylls) and yellow (xanthophylls) colouring matters of leaves, of the red pigments of annatto, the tomato, the Chinese Lantern plant, and the red pepper, of the yellow or orange pigments of carrots, dandelions, sunflowers, saffron, and yellow pansies, or of the brown pigments of some seaweeds; that the molecular configuration responsible for violet and orris perfumes likewise forms a part of the structure of the pigment of the carrot and of vitamin A; or that the colouring matter of egg yolks is a mixture of the yellow pigment found in corn with that which occurs in leaves. Yet this appears to be the case, and all these apparently unrelated substances seem to be built up from the same simple unit, isoprene (C_5H_8), a hydrocarbon heretofore regarded as peculiar to the vegetable kingdom but now shown to play an important role in the animal organism also*[1]

Marston Taylor Bogert (1868-1954)
Organic Chemist and Professor at Columbia University, USA

Volatile isoprenoid emission by plants: Where, what and how?

Plant isoprenoid biosynthesis is one component of secondary metabolism that occurs through one of the two spatially separated pathways within a plant cell (Nagegowda, 2010; Schnitzler et al. 2010). A cyanobacterial pathway that takes place in the plastid, also referred to as DOXP/MEP (1-Deoxy-D-xylulose-5-phosphate/2-C-methyl erthritol-4-phosphate) pathway and an archaeal pathway in the cytoplasm, also referred to as the MVA (mevalonic acid) pathway (Sharkey and Yeh, 2001, Figure 1). Isoprenoids (also called terpenoids) are a large class of versatile macromolecules with great structural diversity

[1] Bogert, M T (1932), Recent Isoprene Chemistry, *Chemical Reviews*, Vol. 10 (2), 265-293

despite being all constructed by catenation of five carbon (C5) monomers that are derivatives of isopentenyl pyrophosphate (IPP) and dimethyalyl pyrophosphate (DMAPP).

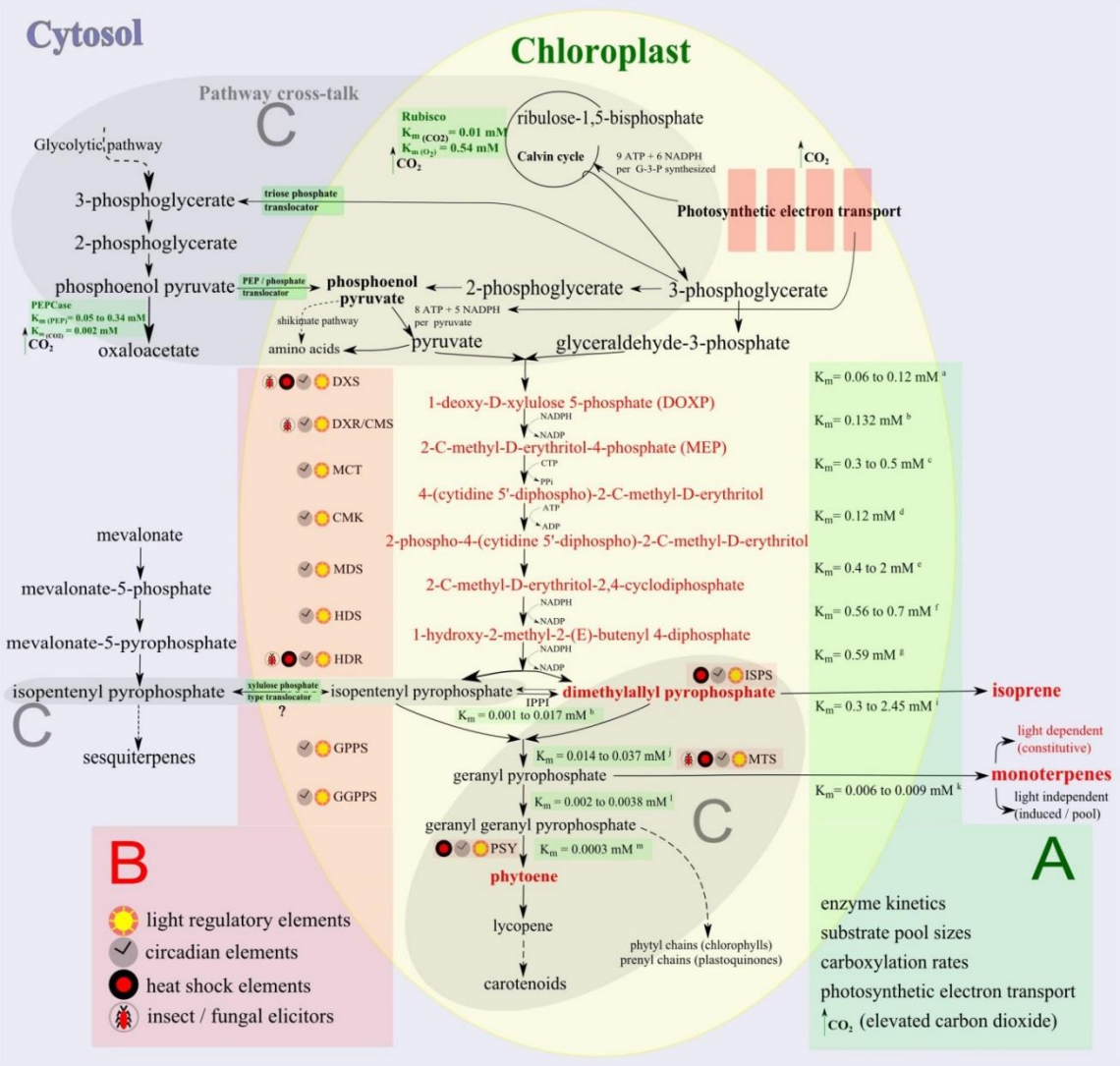

Figure 1: The complex network of biosynthetic pathways closely linked with the MEP pathway (also see figure 3 on page 27 and Appendix I)

(A) ***Constitutive emission*: Substrate pool limitation and enzyme kinetics of the MEP pathway and photosynthesis:** Isoprene (if not monoterpene) emission is shown to be strongly limited by DMAPP pool size at least over short time scales. (B) ***Transient maximum emission*: Transcriptional controls on the pathway network:** Most genes in the plastidic MEP pathway have light-regulated circadian elements (Wilkinson et al. 2006; Cordoba et al. 2009) along with putative heatshock promoter elements upstream of their initiator codon (Sharkey et al. 2008). It is important to note that MEP pathway enzymes are most likely to be available, albeit at very low amounts, even when transcription is not induced as the enzyme half-life of MEP enzymes are >3 to 4 days. An onset of a stress signal such as heat, insect attack, or photo saturation would lead to rapid depletion of MEP pathway substrates via isoprenoid emission. It creates fresh demand for MEP substrates and enzymes. (C) ***Seasonal variability*: Pathway trade-offs:** Mechanisms of components A and B operating over seasonal time scales involve activation of genes from other linked pathways (both within and outside chloroplast) that would affect MEP pathway substrate pools. These changes cause substantial fluctuation in constitutive emission capacities. The transcriptional sensitivity of all the associated genes to photoperiod needs to be parameterized over annual time scales.

The conjugated double bounds (dienes) of volatile isoprenoids make them readily react with any atom/molecule with unpaired valence electrons. These are made by one (isoprene), two (monoterpenes) or three (sesquiterpenes) C5 units. In plants, the enzymes involved in the plastidic MEP pathway are responsible for isoprene and monoterpene emissions. These enzymes nuclear encoded and plastid targeted. It is known that both isoprene and monoterpene biosynthesis principally utilize fresh carbon from primary photosynthates fixed during photosynthesis (*de novo*; Schnitzler et al, 2004). Isoprene and *de novo* monoterpene emissions are shown to increase with increasing temperature and luminosity. Monoterpenes are emitted constitutively as well as stored in specialised glands in the leaves by many plants. Monoterpenes emitted from the stored pools are shown to be temperature dependent and light independent (Kesselmeier and Staudt, 1999).

Why do we study plant volatile isoprenoid emission?

Volatile isoprenoid emission by plants has several fundamental and applied dimensions of great importance to human beings and the planet:

(a) Biogenic isoprenoid emission (1000 TgC/yr) is a significant source of atmospheric carbon and is part of the global carbon cycle (Pacifico et al. 2009). Emissions increase atmospheric lifetime of methane (Monson et al. 2007), a major greenhouse gas. Emissions also mediate secondary organic aerosol formation affecting air quality and rainfall (Claeys et al. 2004; Heald et al. 2005; Paulot et al. 2009).

(b) Oxidation products of isoprene (C_5H_8) and monoterpenes ($C_{10}H_{16}$) have significant influence on tropospheric ozone formation (interactions between anthropogenic nitrogen oxides and reactions of photolytic products of free radicles with isoprene). Anthropogenic sulphur oxides (mainly from coal-powered electricity stations) are known to react with biogenic isoprenoids to form sulphuric acid, which has severe

detrimental impacts on vegetation and human health through acid rains (Mauldin et al. 2012).

(c) Natural rubber (polyisoprene) produced by the tree *Hevea brasiliensis* has a global market that is worth > $US 35 billion per annum and there is an increasing global demand for industrially produced isoprene (used in the production of synthetic rubber) and monitory worth is estimated to be > $US 2 billion per annum (ETC Report, 2014)

(d) Isoprene and mixtures of highly volatile isoprenoids are seen as potential aviation fuels and several labs around the world are involved in scaling up microbial production of isoprene.

(e) Higher order terpenoids such as carotenoids have immense value in food and agricultural industries and their worldwide trade is estimated to be worth nearly $US 1 billion per annum (Dufosse et al. 2005; Ribeiro et al. 2011).

Why do plants emit volatile isoprenoids?

The answer to the question of why plants emit isoprenoids is not simple, and not fully resolved. While functions of monoterpene emission are apparent in pollination ecology and plant defense systems, the role of isoprene emission is unclear. At present several hypotheses are in circulation that try to explain the functional utility of isoprene emission to plants and try to identify the selective forces that led to persistence of emissions. These include isoprene emission-assisted thylakoid membrane stability at high temperature, imparting thermotolerance in plants (Singsaas et al. 1997; Behnke et al. 2007; Velikova et al. 2011) and protection of photosynthetic apparatus through quenching of ozone induced free radicles by isoprene (Loreto and Velikova, 2001; Vickers et al. 2009b). However, isoprene [oid] emission is not the only mechanism that achieves these adaptive roles.

Isoprene could also be seen as an inconsequential product of the pathway when the substrate intermediates are not completely consumed by the photosynthetic apparatus. CO_2-enrichment experiments have led researchers hypothesize that under elevated CO_2, the MEP pathway could experience carbon starvation due to reduced PEP flux into chloroplast (Rosenstiel et al. 2003). ATP and NADPH starvation of the MEP pathway due to enhanced carbon fixation by Rubisco could also explain differences in isoprene emission responses under different conditions (Niinemets et al. 1999) and similar models have been refined and elaborated over the years (e.g. Morfopoulos et al. 2013). Despite significant progress, many deeper questions surround the biochemical regulation of the MEP pathway (Trowbridge et al. 2012; Rasulov et al. 2011; Banerjee et al. 2013).

Why eucalypts?

Transgenic *Arabidopsis thaliana* and *Nicotiana tobacum* have been used in some isoprenoid pathway inhibition studies (Laule et al. 2003) as well as induced thermal tolerance experiments (Loivamaki et al. 2007a; Vickers et al. 2009b). However, *Arabidopsis* and *Nicotiana*, the model organisms for most *in vitro* transgenic experiments, unfortunately are isoprene non-emitters and not ideally suited for investigating the biology of emission coupled with climate change scenarios. Studies on isoprene non-emitting RNAi mutant *Populus* (a model tree genus) overcame some of the limitations of herbaceous models (Behnke et al. 2007; 2009). However, species of poplars and oaks are solely either isoprene or monoterpene emitters making them unsuitable for studying the relative response of isoprene and monoterpenes to environmental changes from a functional and physiological perspective.

Eucalyptus sp., a native Australian tree is known to emit a broad range of volatile organic compounds including isoprene and monoterpenes. Unlike many other widely studied model plants in isoprene research (e.g., *Populus* sp., *Quercus* sp.), *Eucalyptus* is a *dual emitter* in that it emits both isoprene and monoterpenes at measurable levels (He et al. 2000; Loreto and Delfine, 2000; Winters et al. 2009). Many early studies on emissions were carried out on *Eucalyptus* to develop some of the first emission algorithms (Guenther et al. 1991, 1993). Since then, several independent research groups around the world have published isoprenoid emission response of various *Eucalyptus* spp from different environments (Calfapietra et al. 2007; Heald et al. 2009; Wilkinson et al. 2009).

On the biology of eucalypts:

***Phylogeny, diversity and biogeography*:** The genus *Eucalyptus* belongs to the family Myrtaceae and it is among the most diverse angiosperms (e.g. Gill et al. 1985) with nearly 900 described species. There is no consensus view on the classification of eucalypts although most taxonomists now recognize three prominent member genera *Eucalyptus*, *Corymbia* and *Angophora* (amongst others) and the family Myrtaceae has undergone repeated taxonomic revision and continues to change with the advent of more nuclear markers and genome sequence information. The earliest ancestors of eucalypts in the family Myrtaceae most likely had their origins in the Gondwanan Antarctica during the late Cretaceous (80 Mya; Figure 2) and the earliest eucalypts diversified though Oligocene (40 Mya) in Australia and South American land mass, although today they are mostly endemic to Australia (e.g. Dettmann, 1992; Ladiges, 1997). Natural populations of a few species are found in the Malayan archipelago and New Caledonian islands. Eucalypts played and continue to play an integral role in shaping the Aboriginal Australian life. Since the year

1780 onwards (since European settlement in Australia) eucalypts have gradually been introduced to many parts of the world as plantation trees to serve the global timber, paper and pulp industries (Turnbull, 2000).

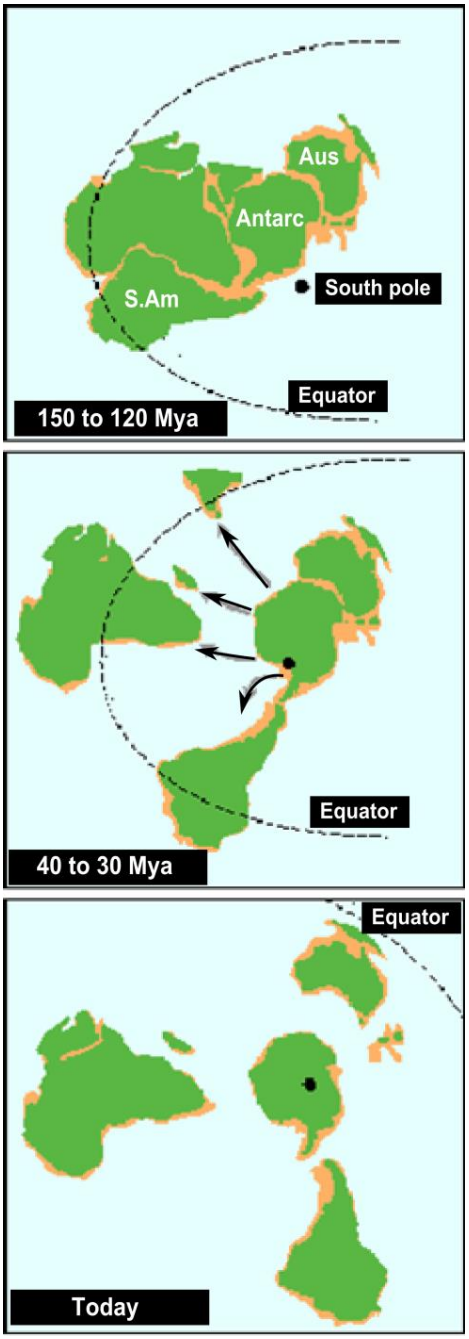

Figure 2: Continental drift in the Southern hemisphere since the late Mesozoic era (150 Mya). Eucalypts were widespread across South America, Antarctica and Australia during their early evolution (60 to 30 Mya) but eventually became isolated in Australia and surrounding islands (today). Illustration is adapted in a modified form from a former webpage of CSIRO, Australia.

***Reproduction and Genetics*:** Eucalypts are generally protandrous outbreeding species. They are pollinated mainly by birds, animals and irregularly by insects (House, 1997). Eucalypts have 11 to 12 pairs (2n = 22 to 24) of small and condensed somatic chromosomes (Matsumoto et al. 2000). The high chromosome numbers may have increased the frequency of meiotic recombination and contributed to their large reproductive success and diversification (Rye and James, 1992; Guerra, 2008). The genome size of eucalypts may vary between 450 and 700 Mb (Grattapaglia and Bradshaw, 1994) and recently sequenced genome of *Eucalyptus grandis* stands at ~640 Mb (Myburg et al., 2014).

***Ecology*:** Despite being genetically very similar, eucalypts exhibit astonishing phenotypic diversity. Several key nucleotide polymorphisms, differences in gene expression and isozymes are responsible for differences in eucalyptus leaf economics (Niinemets et al. 2009), photosynthetic properties (Lewis et al. 2011), flowering time (House, 1997), disease resistance (Potts, 2000), canopy architecture and longevity (Mooney et al. 1978). Eucalypts are mostly arborescent and show remarkable adaptation to some of the harshest habitats in Australia (Hughes et al. 1996). Some species tolerate alpine (<−20 ^{o}C) conditions (e.g. *Eucalyptus archeri*) while some survive in dry and hot deserts (e.g. *Eucalyptus striaticalyx*). The tallest angiosperm known is a eucalypt (*Eucalyptus regnans*). Insects have co-evolved ingenious methods to feed and digest eucalyptus leaves rich in noxious secondary metabolites (Ohmart and Edwards, 1991). The ability of eucalypts to regenerate through epicormic bud sprouts has meant that they withstand insect herbivory that would otherwise completely defoliate even large trees. Epicormic regeneration in eucalypts plays a critical role in the maintenance of fire-prone Australian savannah and sub-tropical woodlands (Burrows, 2002).

***Physiology*:** Eucalypts are fast growing pioneer species that prefer high light environments and have a wide tolerance for water and nutrient availability. They have extraordinary photosynthetic capacities and some species can fix carbon at rates faster than crop plants (Wullschleger, 1993). A latent capacity for high metabolic growth in eucalypts is a paradox given that most eucalypts grow in nutrient poor sub-optimal conditions and as a result their operating photosynthetic rates do not reach their maximum potential (Whitehead and Beadle, 2004). Xylem vessels (>150 µm in diameter) in eucalypts are some of the widest among angiosperms (Mokany et al. 2003; Sperry et al. 2006). As a result, eucalyptus populations are vulnerable to temporary periods of severe heat or drought, which are common in Australia. Yet, they have evolved a wide ranging capacity for transpiration, mechanisms of osmotic adjustments and seasonal modification of hydraulic architecture, all of which help them survive short-periods of extreme stress (e.g. Merchant et al. 2007; Atwell et al. 2009).

Challenges:

***The trouble with global emission models*:** Estimating global biogenic volatile isoprenoid emission levels has been ongoing since the 1960s (Went, 1960a; 1960b). The interest in quantifying emissions arises from the known impacts of biogenic hydrocarbons on tropospheric ozone, NO_x and methane and the roles of emission in plant function. Since the beginning of 1990s, global isoprenoid emission models trying to capture current and future trends in emission levels have involved *a posteriori* approaches involving palaeoclimate and satellite based measurements (Shim et al. 2005; Arneth et al. 2007) as well as process-based emission algorithms integrated with dynamic vegetation models (Guenther et al. 2006; Unger et al. 2013). Interestingly, these modelled estimates, especially for global annual isoprene emission levels, have been fairly consistent (500 ± 50

TgC/yr) and the consistency is attributed to the conformist assumptions of existing emission models (Fehsenfeld et al. 1992; equations and estimates are neatly summarized in Arneth et al. 2008).

Our understanding of the biochemistry and physiology of isoprenoid emission has deepened since the 2000s, but this has in turn led to the requirement for more sophisticated modelling and interpretation. With increasing anthropogenic inputs into the earth's future greenhouse gas budget and increase in global temperatures plant isoprenoid emissions are likely to increase but such an increase is projected to be compensated by a decrease in tropical forest cover (IPCC, 4th AR, 2007). There is a need to design and conduct experiments that comprehensively tackle emission responses by accounting for genetic diversity and species-specific tolerances to abiotic stresses such as heat and drought. There is also a need to adopt simpler mechanistic models that capture important features of emission response to key indicators of climate change.

The role of volatile isoprenoids in emitting taxa: After six decades of research and progress, we are still far from knowing the means of natural selection that has shaped emission capacity in plants and why isoprene emission has evolved only in some plant taxa and not in others (Sharkey and Singsaas, 1995; Gray et al. 2011; Fineschi et al. 2013). Leading researchers appear to have come to a point where they are now questioning the question "why plants emit isoprene?", albeit with some optimism (Sharkey et al. 2013). A justification for asking questions about the evolution of isoprenoid emission could be provided at two levels.

(a) At a practical level, isoprenoid emission is net carbon lost by plants (hence has a metabolic cost) and likely has many functions (Figure 3). A large range in rates of

isoprenoid emission between plant taxa is a big hurdle to improving global isoprenoid emission estimates (see Appendix I). If one assumes that isoprenoid emission has key a role in increasing plant fitness, then understanding evolutionary constraints on the emission process becomes an essential prerequisite for the successful prediction of emission capacity in plants.

(b) At a philosophical level, evolution by natural selection is a feature of the living world that distinguishes it from the physical world.

> "*Because of the cunning shown by natural selection the whole of Nature is little more than a series of gadgets. This distinguishes [it] strongly from almost all the important problems in physics. Typically, the errors in one gadget are corrected in a further one*"
>
> p 165; *Francis Crick, The discoverer of the genetic code*: A biography by Matt Ridley (2006)

Francis Crick (co-discoverer of the DNA double helix), a physicist, is said to have realized the role played by natural selection in living systems through his interactions with biologists such as Jacques Monod (co-discoverer of the *lac* operon). We know in a very crude sense that those 'living gadgets' simultaneously undergo correction of old "errors" (adaptation/selection) and further accumulation of new "errors" (mutation). While laws of physics (particularly the laws of thermodynamics) are very powerful and may explain a lot of what *life* is, it is ultimately an evolutionary context that helps us make sense of biological processes (Dobzhansky, 1973).

***Isoprenoids and human welfare*:** Forests have been emitting volatiles for millions of years but it is only since the industrial revolution and post WW-II urbanization of this world that formation of smog and air quality deterioration has become a major challenge. Plantation strategies involving transgenic isoprene non-emitting trees have been proposed (e.g. Behnke et al. 2012) to curb the impact of isoprene emission on tropospheric ozone

pollution (mediated by NO_x). The challenge also lies in understanding the chemistry of volatile isoprenoid-NO_x-O_3 interactions in real time, particularly in regions where urban areas are in close vicinity of reserved forests.

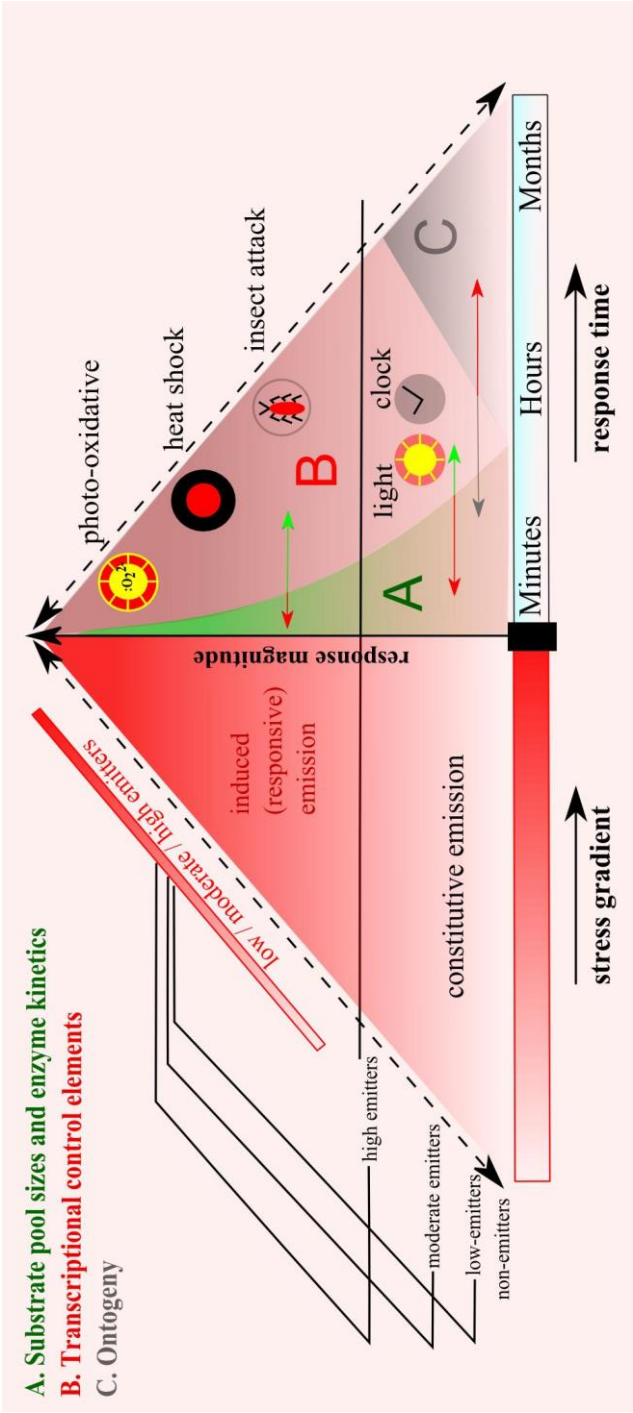

Figure 3: A schematic diagram distinguishing constitutive and induced emission (Harrison et al. 2013, also see Appendix I)

Research questions and thesis structure

In this thesis, I present the results of a comprehensive set of experiments on the physiology of isoprenoid emission in selected species of *Eucalyptus* from different climate zones in Australia. These experiments attempt to generate a physiological and evolutionary basis for isoprenoid emission capacity in plants, with a particular focus on the role of physical factors such as drought, CO_2 and O_2 levels, photoperiod and temperature in regulating isoprene and monoterpenoid emission responses at distinct regulatory hierarchies. Another broad aim was to come up with an overarching hypothesis to explain the mysterious origins and phylogenetically disjunct occurrence of isoprene emission capacity among major groups of land plants. I am avoiding citing papers in this short overview of the thesis structure, since a large number of citations would overwhelm readability. I request the reader to go to the relevant chapter to find the literature of their interest since each chapter has its own exhaustive list of references. I recommend reading a multi-authored *review* published in *New Phytologist* (**Appendix I**) to place my PhD thesis in the context of developments in the field thus far.

The positive correlation between net carbon assimilation rate and isoprenoid emission rate (with a few exceptions) was established long ago and has been confirmed in different plant systems (see **Chapters 2** and **6**). However, the relationship between the two plastidic processes is less clear in plants experiencing severe abiotic stresses (particularly drought), where photosynthesis clearly declines but the response of isoprenoid emission responses are far less predictable. One of the main aims of the PhD was to exploit the diversity in eucalypts to elucidate the finer aspects of the relationship between photosynthetic carbon assimilation and isoprenoid emission in plants experiencing moderate to severe abiotic

stresses (drought, high temperature, low CO_2, and high O_2). Building on a preliminary emission screening elaborated in **Chapter 2** (aimed at describing the bio-geographic considerations that led to species selection in later chapters), I present a substantive argument in Chapter 3 in favour of the reducing power dependent isoprene emission in eucalypts under drought and other forms of abiotic stresses. **Chapter 3** is a full paper published in *Plant Physiology* (published by the American Society of Plant Biologists). Closely linked with Chapter 3 is the subject discussed in **Chapter 4**, where I describe the post-illumination behaviour of constitutive isoprenoid emission in *Quercus ilex* (European Holm oak) and *Eucalyptus camaldulensis* (Australian River red gum) in the backdrop of post-illumination CO_2 burst (photorespiration and dark respiration). The role of photorespiration in influencing the carbon and energy balance between co-localised chloroplastic pathways is central to the arguments presented in Chapters 3 and 4.

While increasing global mean temperatures and increased frequency of unseasonal drought could be two of the most significant drivers of a potentially increasing trend in isoprenoid emission from forests, an important yet often overlooked external factor is *photoperiod*. Photoperiod is a constant factor that exhibits an annual cycle. Separating the interactive effects of temperature and photoperiod on plant metabolism in general has been a question that has bothered many a plant researcher for the good part of last 100 years. In **Chapter 5**, I discuss the results of experiments dealing with the interactive effects of photoperiod and temperature on seasonal variation in isoprenoid emission in selected species of eucalypts. The aim was to also investigate a role played by photoperiod potentially independent of temperature, in influencing seasonal emissions in eucalypts.

The importance of understanding the origin and evolution of isoprenoid emission trait is briefly mentioned in the introduction to the thesis. In **Chapter 6**, I propose an original

evolutionary hypothesis to explain the occurrence and distribution of isoprene emission trait in major land plant linages. The chapter is published as a full-length opinion article in the journal *Trends in Plant Science* (published by the Cell Press). In a short, yet a comprehensive discourse (**Chapter 7**), I highlight some of the most important means of natural selection acting on volatile isoprenoid emission at various levels of living complexity from bacteria to plant populations. Chapter 7 was invited and published in *Journal of the Indian Institute of Science*. I conclude my thesis in **Chapter 8** by doing a brief recap of the background to this thesis, summarising the main findings of my work and its significance in the field of plant isoprenoid research. Exceprts of Chapter 8 are published as an *addendum* in the journal *Plant Signaling and Behavior* (**Appendix VII**). In the concluding chapter, I attempt to identify future directions of research that would help fill the remaining gaps in our knowledge of plant isoprenoid emission and its role in the earth system.

Major milestones in isoprenoid emission research:

1956 Guivi Sanadze (Georgian Academy of Sciences, Georgia) discovers the phenomenon of plant isoprene emission (Sanadze, 1957); Reinhold Rasmussen at Washington University in USA later discovers the phenomenon independently in the 1960s (Rasmussen and Went, 1960)

1979 The positive effect of light and temperature on isoprene emission initially proposed in the 1960s is unequivocally proven by David Tingey (Environment Protection Agency, USA) (Tingey et al. 1979)

1987 Isoprene emission capacity is demonstrated in lower plants (ferns) by David Tingey (Tingey et al. 1987)

1989-1994 Correlation between photosynthesis and isoprene emission is established (Monson and Fall, 1989; University of Colorado, USA) and later a biochemical link is proved by $^{13}CO_2$ labelling (Delwiche and Sharkey, 1993; University of Wisconsin, USA)

First extensive physiological investigations of temperature (Monson, 1992) and drought (Sharkey and Loreto, 1993) effects on emission are reported

MEP pathway is discovered and elucidated in bacteria by Michél Rohmer and others at National School of Chemistry, Mulhouse in France (Rohmer et al. 1993)

1995 The very first isoprene synthase enzyme from poplar is characterised by Gary Silver and Ray Fall at University of Colorado (Silver and Fall, 1995)

Alex Guenther at National Centre for Atmospheric Research (USA) proposes a global emission algorithm (G95) based on empirical evidence. (Guenther et al. 1995)

1997 Origin of isoprene emission in plants is traced to a plastid localised MEP pathway (Lichthenthaler et al. 1997)

Isoprene mediated thermotolerance hypothesis is proposed with experimental evidence (Sharkey and Singsaas, 1995; Singsaas et al. 1997)

1998-2006 First attempts to construct phylogenetic trees of isoprene emitters and non-emitters and hypotheses tackling origin and evolution (Harley et al. 1999; Sharkey et al. 2005)

Isoprene emission mitigating oxidative stress (ozone) in plants receives empirical support (Loreto and Velikova, 2001; The National Research Council, Italy)

Transgenic isoprene emitting *Arabidopsis* are generated (Sharkey et al. 2005; University of Wisconsin, USA)

Genes and enzymes of the MEP pathway are isolated and characterized from *E. coli* and plant systems by researchers from all over the world (e.g. Miller et al. 2001)

Metabolic cross-talk between cytosolic MVA and plastidic MEP pathway is established (Laule et al. 2003; Hammerlin et al. 2003)

The complete MVA pathway construct is expressed in *E. coli* and a template is set for large-scale production of isoprenoids using microbial system (Martin et al. 2003)

Various process-based mechanistic models are proposed to capture isoprene emission behaviour in response to physical factors (Niinemets et al. 1999; Zimmer et al. 2000)

Sophisticated top-down (satellite) and bottom-up (MEGAN) approaches are pursued (Shim et al. 2005; Guenther et al. 2006)

2007-2009 Transgenic isoprene emitting tobacco and isoprene non-emitting poplar systems are developed in the UK (Essex/Lancaster) and Germany (Karlsruhe) (Behnke et al. 2007; Vickers et al. 2011)

A unifying hypothesis regarding the role of plant isoprene emission in mitigating various forms of oxidative stress is proposed (Vickers et al. 2009a)

The role of substrate limitation (DMAPP) in regulating isoprene emission is demonstrated using post-illumination isoprene burst (Rasulov et al. 2009)

2010-2013 Metabolic bottle-necks and pathway feedback mechanisms in the MEP pathway are discovered (various authors)

More hypotheses concerning origin and evolution of plant isoprene emission are proposed (Sharkey et al. 2012; Monson et al. 2013)

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Chapter 2

Photosynthesis and isoprene emission in eucalypts from contrasting environments[2]

[2] Contributions: This chapter emanates from a collective discussion and decision on experiments by all three supervisors of KGSD and him. IMJ and KGSD designed the canopy chamber and sampling unit. METS engineers constructed it. KGSD carried out all the experiments and analyzed the data and wrote it in a manuscript format. The process based emission model was developed by a team of researchers led by ICP (see Appendix I).

Abstract:

Isoprene biosynthesis and emission by plants depends on carbon, energy and reducing power supplied by photosynthesis. Experiments have established a negative relationship between leaf-internal CO_2 concentration (c_i) and isoprene emission rate (I_e) within individual species, in contrast to the positive relationship between c_i and photosynthesis. In this study, we exploited natural variation in c_i:c_a ratios among eucalypt species from diverse environments by examining the relationship between net carbon assimilation rate (NAR) and isoprene emission *between species* under present-day ambient CO_2 concentration (c_a=400 ppm). A_{sat} varied greatly, and was a strong predictor of I_s among species (r^2=0.72, P<0.01) whereas I_s did not correlate with c_i. Among species with adult foliage, A_{sat} was co-limited by RuBP carboxylation and regeneration. The ratio of electron transport rate (J) to carboxylation capacity correlated *negatively* with I_s while the proportion of electron flow not used in the dark reaction (J_r) was *positively* correlated with I_s (r^2=0.65, P=0.05). Juvenile foliage of two dimorphic species were outliers, showing high I_s relative to A_{sat}. We hypothesize that a disequilibrium between electron transport rate and Rubisco capacity in some plants may contribute to their high isoprene emission capacity.

Key words: CO_2 acclimation; isoprene emission; MEP pathway; electron transport; photosynthetic optimum

Introduction:

Photosynthesis, inferred to be optimally co-limited by Rubisco (A_c) and RuBP regeneration (A_j), is the principal contributor of carbon skeletons and reducing power used in volatile isoprenoid biosynthesis, at least under stress-free conditions (Loreto et al. 1996; Sharkey and Yeh, 2001; Schnitzler et al. 2004). Isoprene emission rate (I_e) increases with increasing light and temperature and consideration of CO_2 effects is important as CO_2 strongly opposes the effect of temperature (Possell et al. 2005). The effect of CO_2 concentration on emission has been widely studied, in part because of the central role that changes in CO_2 concentration have played in the evolution of photosynthetic metabolism in land plants. *Eucalyptus* is a native Australian tree genus encompassing a large and diverse clade (~1000 species) of plants that emit a broad range of volatile isoprenoids such as isoprene and some monoterpenes synthesized *de novo*; and several monoterpenes from

stored pools (e.g. Winters et al. 2009). Exploiting the natural variation in c_i:c_a ratios in eucalypts, we tested whether relationships between photosynthesis and isoprene emissions are conserved across different species with distinct adaptations, some characterized by distinct juvenile foliage during early development. Juvenile foliage of eucalypts is physiologically and morphologically mature yet it is distinct from adult foliage (e.g. James et al. 1999, Velikova et al. 2008) and the duration of juvenile phase varies from species to species.

It is known that higher light-saturated carbon assimilation rates of leaves generally are associated with higher isoprenoid emission capacity within species (Monson and Fall 1989). We started with an assumption that high net assimilation rate (NAR) reflects a large *de novo* substrate pool and high NAR likely co-occurs with "an equally active" MEP pathway even across species. We hypothesized that a potential disequilibrium between carboxylation and photosynthetic electron transport capacities in some plant species (in favour of ETR) could explain their unusually high constitutive volatile isoprenoid emission capacities.

Materials and methods (also see Appendix II and III):

Plant material: Seeds of five species of the genus *Eucalyptus*, namely *E. striaticalyx*, *E. camaldulensis* (including three subspecies), *E. dunnii*, *E. haemastoma* and *E. globulus*, and one species each of the genus *Corymbia* (*C. tessellaris*) and *Angophora* (*A. costata*) were obtained from the Australian Tree Seed Centre (Canberra). The selection of species was based on their phytogeographic distribution representing different climatic zones in Australia (Figure 1). Soil (a Krasnozem from Robertson, NSW) was mixed with mineral

nutrients (Table S1; Appendix III) during germination and early growth. Seeds were germinated in winter (May 2011) at 25 °C day/18 °C night under natural photoperiod in a glasshouse. Later *Osmocote*® slow release fertilizer (0.5% of dry weight of soil) was mixed with 80 kg soil in large pots and germinated seedlings were transplanted into these pots and grown under ambient CO_2 (400 ± 50 ppm); temperature of 29 °C day/21 °C night) in a glass house. Eight-month-old potted seedlings were used for volatile sampling.

Estimating photosynthetic capacities: Photosynthesis was monitored using a LiCor 6400XT portable photosynthetic system between 12 noon and 3 pm, at a leaf temperature of 30 °C, relative humidity of 40 to 65%, and a light intensity of 1500 µmol m^{-2} s^{-1} on bright sunny days during March/April 2012 (autumn). *A*-c_i curves were fitted and V_{cmax} and *J* were estimated using the curve-fitting tool described in (Sharkey et al. 2007); see Tables 1 and 3; J_r (electron transport rate not used for dark reactions of photosynthesis, given c_i and V_{cmax}) was calculated following Harrison et al (2013).

$$J_v = V_{cmax}\left[\frac{c_i + 2\Gamma^*}{c_i + K_m}\right] \quad (1)$$

$$J_r = (J - J_v) \quad (2)$$

Given V_{cmax}, we calculated J_v and then substitute (1) in (2) where,

J_v = proportion of electron transport used for dark reactions; V_{cmax} = maximum carboxylation rate by Rubisco; K_m = effective Michaelis-Menten coefficient for carboxylation by Rubisco (1100 ppm at 30 °C); Γ^* = photorespiratory compensation point (40 ppm for C_3 plants, although it varied across species); c_i = species-specific leaf internal CO_2 concentration (ppm) at ambient CO_2 = 400 ppm.

***Volatile isoprenoid sampling*:** A 250 L capacity whole plant canopy cylindrical volatile collection chamber (see Appendix II) was assembled using volatile free FEP

fluorocarbon film (200 µm thickness, transparent to solar spectrum; Dupont ™) and the gas-exchange line was made of Teflon, stainless steel tubing and Swagelok® connectors. High purity instrument grade air (BOC; 78% nitrogen, 21% oxygen, 1% argon) was mixed with CO_2 (5% in Nitrogen) to achieve ambient CO_2 concentration in the head space. Head space flushing and blank samplings were run to eliminate memory effects (after Niinemets et al. 2011). Soil emissions were prevented from entering the head-space by a Teflon cover sealed around the base of plant stem. Sampling was carried out between 12 and 3 pm such that they correspond to the time of day when photosynthesis measurements were made. The chamber temperature was 33.3 ± 1.6 ˚C with some exceptions (Table S2, Appendix III), relative humidity generally varied between 25 and 40% (Table S2, Appendix III). Plants were provided with natural PAR of 550 to 800 µmol m^{-2} s^{-1} and supplemented with up to 400 µmol m^{-2} s^{-1} of artificial light such that the incident light intensity striking the canopy chamber exceeded 800 µmol m^{-2} s^{-1} in all cases (often >1000 µmol m^{-2} s^{-1}) while volatile sampling was being carried out. Isoprene emission rate increases with increasing light intensity and any increase beyond ~800 µmol m^{-2} s^{-1} is shown to have no significant impact on I_e (Guenther et al. 1991), although it has to be qualified that for eucalypts even 1500 µmol m^{-2} s^{-1} is not enough to cause light-saturation. Emission responses at the light-level we provided at the canopy crown could only be used as indicative of better-controlled cuvette-based measurements (done in later experiments). Isoprene was adsorbed onto conditioned Carboxen 1016 (Sigma Supelco®) into glass thermal desorption tubes and then analysed off-line by Thermal Desorption-Gas Chromatography Mass Spectrometry (TD-GCMS).

Despite limitations of matching leaf-level photosynthesis and canopy-level emission measurements, we opted for a canopy-level whole plant sampling because it was important to mimic a realistic field setting during emission estimation. Our method would not have

been applicable in species with dense and closed canopies, which eucalypts are not. Quantifying species-to-species variation in the MEP pathway capacity (maximum emission potential) was beyond the scope of this study.

***TD-GCMS analysis*:** A customized GCMS-QP2010 (Shimadzu) was regularly calibrated using standard isoprene (Sigma) samples. Isoprene was diluted in nitrogen filled sterile Tedlar® bags and then adsorbed onto TD tubes. Isoprene sampled from the plant chamber was desorbed at 220 °C with ultrapure helium (BOC) as the carrier gas and cryo-focussed in a stainless steel loop immersed in liquid nitrogen. The loop was re-heated to 260 °C to enable sample injection through a PTV injection port. A 30m, 25mm ID, 25µm RTX-5SilMS (Restek) capillary column was used for GC separation. The chromatographic peaks were identified by comparing them to isoprene standard (m/z = 67). Isoprene emission rates were calculated by utilizing sampling flow rate, quantified standards and total leaf area (TLA). TLA was calculated manually by graphing several representative fully expanded leaf boundaries onto paper and multiplying the average area with the total number of fully expanded mature leaves in the headspace. Monoterpene emissions that were detected were subsequently identified in some species using mass spectra in the NIST library (2008) but none was quantified.

Statistical analyses: Leaf-level photosynthetic measurements corresponding to ambient CO_2 involved several within plant replicates (Table S3). Isoprene was sampled with two to three biological replicates (whole plants) and multiple technical replicates (Table 2) at the same time of day repeated over many days. A-C_c curves were done in independent triplicates using fully expanded leaves. Correlation coefficient and linear regression fit between the variables of interest were obtained and their statistical significance was tested using Minitab16 (Minitab Inc, PA, USA).

Results (also see supplementary tables in Appendix III):

Figure 1:

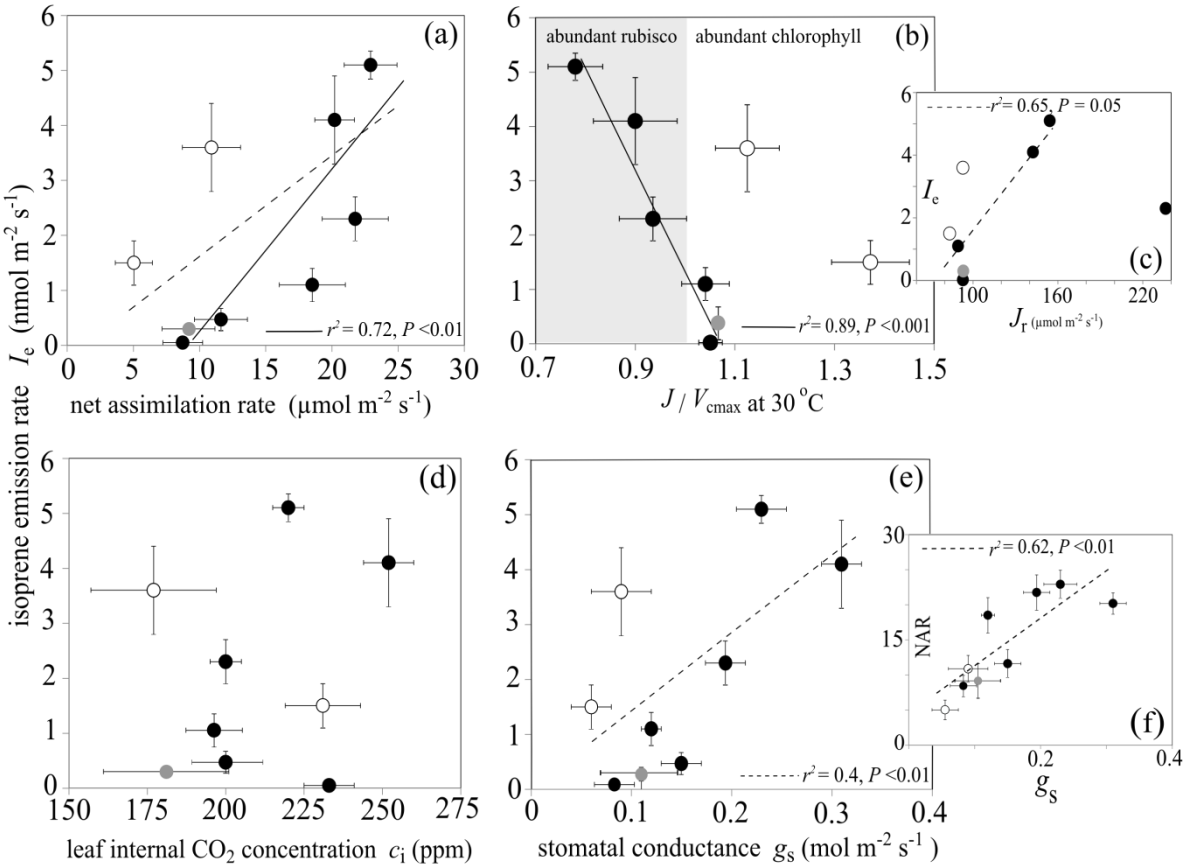

Figure 1: Relationship between (a) NAR; (b) J/V_{cmax}; (c) J_r; (d) c_i; (e) g_s and isoprene emission rate (I_e). (f) g_s vs. NAR. Dotted fit represents overall trend inclusive of all species. Black circles and solid line (species with adult foliage); hollow circles (juvenile); gray circle (*E. haemastoma*). Figure 1(b) and (c) do not have *Angophora costata* since *A-ci* curves were not obtained for that species. The regression fit in Figure 1(c) excludes one point (at the extreme right) *E. camaldulensis* subsp *subcineria*. *Eucalyptus haemastoma* had juvenile foliage that consistently followed the adult foliage regression in all plots (distinguished with a gray circle).

Net carbon assimilation rate (NAR) at an ambient CO_2 of 400 ppm varied from 5 to 22 µmol m^{-2} s^{-1} and NAR was a strong predictor of isoprene emission rate (I_e) among species (r^2 = 0.72, P <0.01), although the relationship was weak when species with juvenile foliage were included (r^2 = 0.34, P = 0.06). Two dimorphic species with juvenile foliage were outliers, showing high I_e relative to NAR. Isoprene emission rates (I_e) varied between

negligible/no emission in *E. dunnii* to 5.1 ± 0.15 nmol m^{-2} s^{-1} in *E. camaldulensis* subsp. *camaldulensis* (Fig 1(a)). Leaf-internal CO_2 concentration (c_i) bore no relation to I_e (Fig 1(d)) while stomatal conductance (g_s) correlated positively with I_e (Fig 1(e)). Stomatal conductance (g_s) ranged from 0.05 to 0.32 mol m^{-2} s^{-1}, and significantly affected photosynthetic rate (Fig 1(f), r^2 = 0.62, P <0.01) but bore no correlation with c_i across species.

Figure 2:

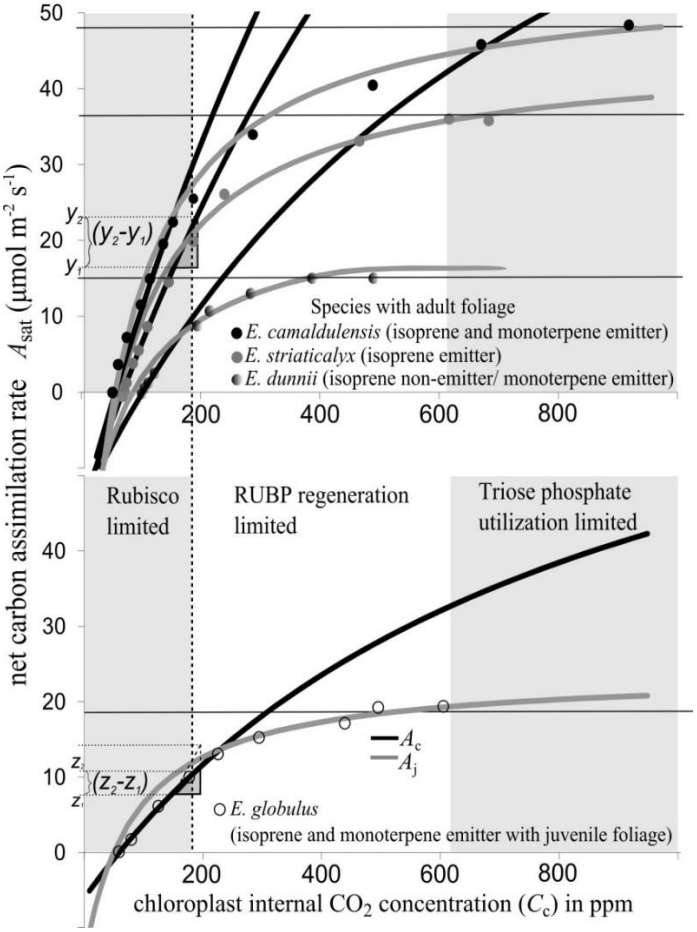

Figure 2: *A*-c_i curves for eucalypts with four unique combinations of constitutive emission capacity. The three zones of limitations on photosynthesis are highlighted (also see Sharkey et al. 2007). The vertical dotted line is a representative chloroplast CO_2 concentration c_i (180 ppm) corresponding to the ambient CO_2 (400 ppm) at which I_e was measured for all species. Note the shallower slope of the curve for *Eucalyptus globulus* e.g. $(z_2-z_1) < (y_2-y_1)$, clearly indicating Rubisco-limited photosynthesis at ambient c_a while photosynthesis in all other species with adult foliage (upper panel) was co-limited by Rubisco and RUBP regeneration.

The ratio of instantaneous electron transport rate (J) to maximum carboxylation rate (V_{cmax}) at 30 °C was negatively correlated with I_e (Fig 1(b); excluding juvenile species); while J_r (J not used for carboxylation) was positively correlated with I_e ($r^2 = 0.65$, $P = 0.05$; all species apart from one adult outlier). NAR in all eucalypts (except *E. globulus* and *C. tessellaris* with juvenile foliage) was co-limited by carboxylation and electron transport ($A_c \approx A_j$) at ambient CO_2 levels during the volatile sampling period (Fig 2).

Discussion:

When compared to published eucalypt emission data (e.g. Loreto and Delfine, 2000; Velikova et al. 2008, Winters et al. 2009) our estimates of I_e for the selected species are conservative. We see fuor reasons. (i) Our emission estimates were derived from whole-canopy sampling and averaged for the total leaf area in the collection chamber. Integrated estimates of gas emission would be lower than peak rates for individual leaves obtained using a LiCor cuvette. (ii) Eucalypts also emit monoterpenes, which are largely stored (*cf.* emissions synthesised *de novo*). Monoterpenes were not monitored in our study because it was a preliminary screening exercise (protocols were still being standardised and optimised). (iii) It was also rationalised that while monoterpene biosynthesis is light-dependent, monoterpene release from stored pools in eucalypts is independent of light. Hence, monoterpene emission was obscurely related to photosynthetic variables. In addition, the data of Kuhn et al. (2002) indicate that the constitutive (non-stored, more volatile) monoterpenoid emission rates would be at least one order of magnitude less than isoprene emission rate and were unlikely to have influenced the emission relationships reported here. (iv) The emission estimates from many other eucalypts done using a canopy chamber very similar to the one we designed, concurred with our estimates and were within

expected range (scientists at CSIRO and Prof. Peter Nelson, *personal communication*). After several experiments (done much later), we were confident that eucalypts are generally very low emitters of isoprene compared to other deciduous high-emitters of Europe (reasons discussed in the *addendum* submitted to *Plant, Signaling and Behaviour*).

The lack of a relationship between c_i and emission levels across species (Fig 1(d)) differs from the consistent negative correlation between the two variables reported at individual leaf level in many species (Possell et al. 2005; Guidolotti et al. 2011). The lack of any relationship between c_i and I_e between species should not come as a surprise since c_i depends on many other complex variables such as leaf anatomy. The positive correlation between stomatal conductance (g_s) and emission across species reflects the association of g_s with NAR (Fig 1(e, f); e.g. Wong et al. 1979), since g_s has no direct bearing on emission (Niinemets et al. 2004).

The negative slope of the relationship between J/V_{cmax} and emission rate (Fig 1(b)) may indicate that a small increment in I_e across species requires a proportionately larger gain in J in order to support both high assimilation rates and provide an excess of electrons to sustain higher emission rates (J_r in Fig 1(c)). In contrast, high amounts of chlorophyll per leaf volume in juvenile leaves of *E. globulus* compared with adult leaves (James et al. 1999) suggest that surplus energy/reducing power is available in juvenile leaves in relation to V_{cmax}. However, high chlorophyll content in an emitting species does not necessarily translate into high isoprene emission capacity. Rubisco-limited photosynthesis in highly irradiated juvenile *E. globulus* (J/V_{cmax} = 1.12; also Fig 2) is not analogous to RuBP regeneration-limited photosynthesis in low-light acclimated (shaded and low-emitting) leaves, although both systems are characterised by high chlorophyll content. This

highlights the role played by physiological adaptations of a species to its ecological niche in determining its emission rates. In most instances juvenile and adult foliage of eucalypts experience contrasting environments (e.g. high vs. low light). The two outliers in Figure 1(a), *Corymbia tessellaris* and *Eucalyptus globulus*, are distinguished by having juvenile foliage which shows relatively high isoprene emission proportional to a moderately low NAR and exhibited large variation in c_i. Examining such exceptions could provide further clues to the interaction between photosynthesis and isoprenoid emission.

Conclusions:

The generally observed equitable allocation of nitrogen into Rubisco and the photosystem proteins in C_3 leaves (Evans and Poorter 2001), and the co-limitation of photosynthesis ($A_c \approx A_j$) under steady-state conditions, do not hold for all C_3 leaves. Our results imply that some high isoprenoid-emitting species may be high emitters because they deviate from steady-state acclimation and co-limitation of photosynthesis. **We hypothesize that disequilibrium between electron transport rate and Rubisco capacity in some plants may contribute to their high isoprene emission capacity.** High-emitting juvenile foliage of some eucalypts provides an example of a transient plant developmental phase that does not maintain a steady-state during early establishment. Plants exposed to prolonged periods of abiotic stress, including drought, can also show photosynthetic disequilibrium that negates acclimated responses of isoprenoid emission (Niinemets et al. 2002, Loreto and Schnitzler 2010). Such instances underline the importance of testing the response of isoprenoid emission to changes in CO_2 and abiotic stresses in species that do not appear to show 'optimal' photosynthetic allocation in natural environments.

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Chapter 3

Increased ratio of electron transport to net assimilation rate supports elevated isoprenoid emission rate in eucalypts under drought[3]

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[3] **Contributions**: Taking a cue from Chapter 2 and discussion in Appendix I, KGSD proposed the hypothesis that an imbalance between light reactions of photosynthesis and carbon reduction cycle under drought could influence isoprenoid emission rate. KGSD designed the experiment with inputs from BJA and IMJ. KGSD carried out all the experiments with some logistic support from Shuangxi Zhou (a PhD student at MQ). KGSD analyzed the data and independently wrote the manuscript with some editorial inputs from BJA, IMJ and ICP. The process based emission model was developed by a team of researchers led by ICP (see Appendix I) and later revised by Morfopoulos et al (2014, *New Phytologist*) cited in our paper. KGSD obtained additional experimental data, revised the manuscript and wrote the responses to manuscript referees and the editor of *Plant Physiology*.

Increased Ratio of Electron Transport to Net Assimilation Rate Supports Elevated Isoprenoid Emission Rate in Eucalypts under Drought[1][W][OPEN]

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Plants undergoing heat and low-CO_2 stresses emit large amounts of volatile isoprenoids compared with those in stress-free conditions. One hypothesis posits that the balance between reducing power availability and its use in carbon assimilation determines constitutive isoprenoid emission rates in plants and potentially even their maximum emission capacity under brief periods of stress. To test this, we used abiotic stresses to manipulate the availability of reducing power. Specifically, we examined the effects of mild to severe drought on photosynthetic electron transport rate (ETR) and net carbon assimilation rate (NAR) and the relationship between estimated energy pools and constitutive volatile isoprenoid emission rates in two species of eucalypts: *Eucalyptus occidentalis* (drought tolerant) and *Eucalyptus camaldulensis* (drought sensitive). Isoprenoid emission rates were insensitive to mild drought, and the rates increased when the decline in NAR reached a certain species-specific threshold. ETR was sustained under drought and the ETR-NAR ratio increased, driving constitutive isoprenoid emission until severe drought caused carbon limitation of the methylerythritol phosphate pathway. The estimated residual reducing power unused for carbon assimilation, based on the energetic status model, significantly correlated with constitutive isoprenoid emission rates across gradients of drought ($r^2 > 0.8$) and photorespiratory stress ($r^2 > 0.9$). Carbon availability could critically limit emission rates under severe drought and photorespiratory stresses. Under most instances of moderate abiotic stress levels, increased isoprenoid emission rates compete with photorespiration for the residual reducing power not invested in carbon assimilation. A similar mechanism also explains the individual positive effects of low-CO_2, heat, and drought stresses on isoprenoid emission.

The emission of volatile isoprenoids by plants (globally amounting to approximately 1,000 TgC year^{-1}, of which isoprene constitutes approximately 500 TgC year^{-1}) plays a significant role in tropospheric oxidation chemistry (Amann et al., 2008). Plant isoprenoid emission at the ecosystem scale is determined not only by intrinsic biochemical and physiological controls but also by the relative abundances of species, each with characteristic baseline emission capacities and each subject to modification by environmental conditions (Harrison et al., 2013). While the effects of most environmental factors on isoprenoid emission have been documented (Loreto and Schnitzler, 2010), their interactions are likely to be complex and hold the key to accurate projections of global emissions (Arneth et al., 2007; Squire et al., 2014). The effect of soil water availability on plant volatile isoprenoid emission is crucial to the projections, especially as rainfall patterns are themselves subject to the impacts of climate change.

Volatile isoprenoid emission is notably insensitive to moderate drought (when the fraction of available soil water ranges from 40% to 70%; Fortunati et al., 2008; Centritto et al., 2011). Given the various other consequences of drought for plant function, such as stomatal closure leading to reduced photosynthesis (Lawlor and Cornic, 2002), increased leaf temperature (Jones, 2004), leaf shedding (Tyree et al., 1993), reduced growth and potential hydraulic failure (Maherali et al., 2004), reduced shoot-to-root ratio (Poorter et al., 2012), increased oxidative stress due to the activation of reactive oxygen species (Mittler and Zilinskas, 1994), and an increased Suc-to-starch ratio, affecting osmotic adjustment (Chaves, 1991), it is not surprising that there are large variations in experimental and field measurements of isoprenoid emission in response to drought (for review, see Laothawornkitkul et al., 2009; Niinemets, 2010).

Isoprene emission involves an energy-intensive biosynthesis through the methylerythritol phosphate (MEP) pathway in chloroplasts (for review, see Sharkey and Yeh, 2001). Photosynthesis contributes the required carbon skeletons and reducing power for isoprenoid biosynthesis, at least under stress-free conditions (for review,

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Table I. *Major sinks for energy and reducing power generated by the light reactions of photosynthesis*

RuBP, 10 Ribulose 1,5-bisphosphate; PCO, photosynthetic carbon oxygenation cycle; PG, phosphoglycolate; H_2O_2, hydrogen peroxide; Asc, ascorbate; MDA, monodehydroascorbate.

Biochemical Pathway	Key Steps	ATPs	NADPHs (or Equivalents)	Reference	Percentage Quantum Share[a]
Calvin cycle or photosynthetic carbon reduction cycle (without photorespiration)	10 RuBP + 10 CO_2 → 10 reduced carbon + 10 RuBP	30	20	Ogren (1984)	49–53
Photorespiration (PCO cycle, including RuBP recovery)	10 RuBP + 10 O_2 → 10 PG + (10 PGA) 10 PG → 10 Glycolate →...→10 Gly→ 5 Ser + 5 CO_2 5 Ser →... → 5 Glycerate → 5 PGA + (10 PGA) → 7.5 RuBP	47.5 (17.5)	30 (10)	Peterhansel et al. (2010)	23–29[b]
Nitrate reduction[c] (photoassimilation)	NO_3^- → NO_2^- → NH_4^+ NH_4^+ + Glu → Gln Gln + 2 oxyglutarate → 2 Glu	1	10	Noctor and Foyer (1998)	2–5
Carbohydrate biosynthesis	6 CO_2 → $C_6H_{12}O_6$ …→ $C_{12}H_{24}O_{12}$	19	12	Skillman (2008)	6–10
Mehler reaction[d] (water-water cycle)	10 water + 5 O_2 → 10 H_2O_2 10 H_2O_2 → 20 Asc → 20 MDA + 20 H_2O 20 MDA + 10 H_2O → 5 O_2	0[d]	10	Heber (2002)	3–13
Other reducing sequences (including lipid biosynthesis, sulfate reduction, and the MEP pathway)		20	10		2–6
Total		~90	72		~100

[a]From Haupt-Herting and Fock (2002) and Skillman (2008). The share is determined by not only the absolute demand per pathway but also the actual instantaneous rates of each process (e.g. nitrate reduction needs a lot of reducing power, but its actual processing rate is very slow compared with core reactions of photosynthesis). If one assumes a linear ETR of 100 μmol m^{-2} s^{-1}, then approximately 50 μmol m^{-2} s^{-1} would be spent on reducing carbon (net assimilation rate of approximately 12 μmol m^{-2} s^{-1}, given that four electrons are needed per 1 mol CO_2 fixed). Similarly, photorespiration accounts for approximately 25 μmol m^{-2} s^{-1}, and the remaining processes utilize 25 μmol m^{-2} s^{-1}. [b]The PCO cycle (per se) requires 17.5 ATPs and 10 NADPH equivalents when we discount the energy consumed by the photosynthetic carbon reduction cycle during RuBP recovery via the photorespiratory route. Therefore, the percentage quantum share of the PCO cycle is roughly half that of the Calvin cycle. [c]Both photorespiration and nitrate reduction have access to reducing power from extraplastid sources. [d]The Mehler reaction utilizes NADH instead of NADPH. It does not consume ATPs but rather adds to the pool of ATPs (Heber, 2002).

see Loreto and Schnitzler, 2010). The photosynthetic energy and reducing power output of a chloroplast are shared in unequal proportions among colocalized pathways (Table I). Under favorable conditions, photosynthetic carbon reduction is the largest energy sink (approximately 50%). Abiotic stresses enhance the supply of energy and reducing power to nonphotosynthetic carbon reduction sinks in the chloroplast (Haupt-Herting and Fock, 2002). Examples include (1) increased photorespiration under drought, at least until Rubisco is directly affected by stress (Lawlor, 1976; Noctor et al., 2002), (2) increased photorespiration under low-CO_2, low-light, or high-light stress (Kozaki and Takeba, 1996), and (3) increased photoreduction of oxygen (O_2) under photooxidative stress (Makino et al., 2002). It has been posited that isoprenoid emission is a mechanism to consume surplus reducing power in stressful high-light and/or low-CO_2 environments (Niinemets et al., 1999; Way et al., 2011). Increased accumulation of secondary metabolites such as phenols, alkaloids, and isoprenoids has been documented in plants under abiotic stresses (for review, see Wilhelm and Selmar, 2011). Decreased carboxylation and increased oxidative stress due to the oversupply of reducing equivalents is seen as the main driver of increased secondary metabolism under drought (Selmar and Kleinwächter, 2013). The postillumination behavior of isoprene emission (primary and secondary bursts) under O_2-free, pure nitrogen (N_2) atmospheres have been attributed to the availability of reducing equivalents (Rasulov et al., 2011; Li and Sharkey, 2013). It has further been proposed that the MEP pathway competes with other sinks for reducing power, so that the flow of reducing power to isoprenoid biosynthesis is proportional to the energy unused for primary metabolism (Morfopoulos et al., 2013, 2014; Dani et al., 2014). However, the MEP pathway's requirement for reducing power is very small relative to that of photorespiration (Sharkey et al., 2008), and given the diversity in the relative sink strengths of intraplastidic processes (Table I), it is still unclear how the demands of these different processes influence one another.

Eucalypts have been used as model systems to study plant isoprenoid emission since the early years of isoprenoid research (Guenther et al., 1991; Brilli et al., 2013). All eucalypts store monoterpenes as well as constitutively emit isoprene and some monoterpenes (He et al., 2000). *Eucalyptus camaldulensis* subsp. *camaldulensis* (river red gum) is a drought-avoiding mesic species that is tolerant to waterlogging and distributed in riparian, temperate southeastern Australia (Farrell et al., 1996). *Eucalyptus occidentalis* (swamp yate) is a drought-tolerant species found in saline environments in Mediterranean southwestern Australia (Benyon et al., 1999; Searson et al., 2004). *E. camaldulensis* subsp. *obtusa* is the most widespread eucalypt in subtropical Australia (Butcher et al., 2009). Exploiting this ecological contrast, we empirically tested the hypothesis that constitutive isoprenoid emission is driven by ATP and NADPH availability (Loreto and Sharkey, 1993; Niinemets et al., 1999) and could potentially compete for the same with carbon assimilation (Harrison et al., 2013; Morfopoulos et al., 2014). We manipulated the energy source-sink dynamics by imposing various abiotic stresses, including drought, heat, low CO_2, and high O_2. We investigated the relationship between three plastidic biochemical processes (carbon assimilation, photorespiration, and volatile isoprenoid emission) in eucalypts acclimated to drought for 4 to 6 months. Interactive effects of short-term exposure to five CO_2 concentrations, three O_2 levels, and heat stress on isoprenoid emission rates were also analyzed. It was hypothesized that the relative sink strength of various processes requiring reducing power in the chloroplasts could determine the variations of isoprenoid emission in plants experiencing abiotic stress. We started with the premise that the light-dependent and light-independent reaction components of photosynthesis have different susceptibilities to abiotic stress (particularly to drought) and that these susceptibilities vary across species.

RESULTS

The results are from three independent experiments (see Table II).

Photosynthesis

Acclimation to Drought in Paired Species (at 20% O_2)

E. occidentalis had a significantly higher stomatal conductance (g_s; $P = 0.043$) when watered to field capacity (FC) than *E. camaldulensis* subsp. *camaldulensis*, but the difference in transpiration rate (T_r) was not significant ($P = 0.193$). Net assimilation rate (NAR) of both *E. occidentalis* (16.8 ± 2.28 μmol m^{-2} s^{-1}) and *E. camaldulensis* subsp. *camaldulensis* (18.1 ± 1.86 μmol m^{-2} s^{-1}) was comparable (test of equal means, $P = 0.001$). During acclimation to severe drought stress (FC $\leq$ 50%), *E. camaldulensis* subsp. *camaldulensis* showed a significant decline in all photosynthetic parameters ($P < 0.001$), whereas net assimilation in *E. occidentalis* (15.3 ± 1.81 μmol m^{-2} s^{-1}), although decreased, remained comparable to control values despite a significant decrease in g_s (Fig. 1, A and D). Under well-watered conditions, estimates of photosynthetic linear electron transport rate (ETR) based on chlorophyll fluorescence for *E. occidentalis* and *E. camaldulensis* subsp. *camaldulensis* showed a consistent proportionality with their respective NARs. For *E. occidentalis*, the ETR-NAR ratio remained unchanged even at 50% FC, while the ratio increased significantly (doubled) for *E. camaldulensis* (Fig. 2A). The carbon cost of isoprenoid emission as a proportion of net assimilation increased more than 10-fold as drought intensified and was highest in both species at 25% FC (Fig. 2B). ETR was measured independently using fluorescence and was not coupled with LiCor measurements (experiment 1; Table II). Hence, the observations should be treated only as indicative and not as absolute. ETR-NAR ratios (approximately 7:1 under 20% O_2) were more realistic when obtained after fitting A-C_i curves (experiment 3; Supplemental

Table II. *Experimental layout*

Experiment	Focus	Species	I_e:M_e Molar Ratio	Drought Acclimation	CO_2 Concentration		Temperature	O_2	
					Growing Condition	Exposure before and during Sampling		Growing Condition	Exposure before and during Sampling
					$\mu mol\ mol^{-1}$		°C		%
1. Paired set (Fig. 1)	Effect of drought tolerance of photosynthesis on isoprenoid emission	*E. occidentalis* (drought tolerant)	10:1	100% FC					
(Fig. 2)		*E. camaldulensis* subsp. *camaldulensis* (drought sensitive)	10:1	70% FC (3 months)	400	400	25	20	2, 20
(Fig. 4)				50% FC (15 d) 25% FC (15 d)					
2. Individual species (Fig. 3)	Interactive effects of heat, CO_2, and drought on isoprenoid emission	*E. camaldulensis* subsp. *obtusa*	2:1	100% FC	400		28	20	2, 20
				50% FC (1 month)		60, 180, 400, 1,000, and 1,800	38 (Only exposure)		
3. Individual species (Fig. 5)	Relationship between photorespiration, net assimilation, and isoprenoid emission	*E. camaldulensis* subsp. *camaldulensis*	10:1	100% FC	400	400	25	20	2, 20, and 50
				50% FC (10 d)					

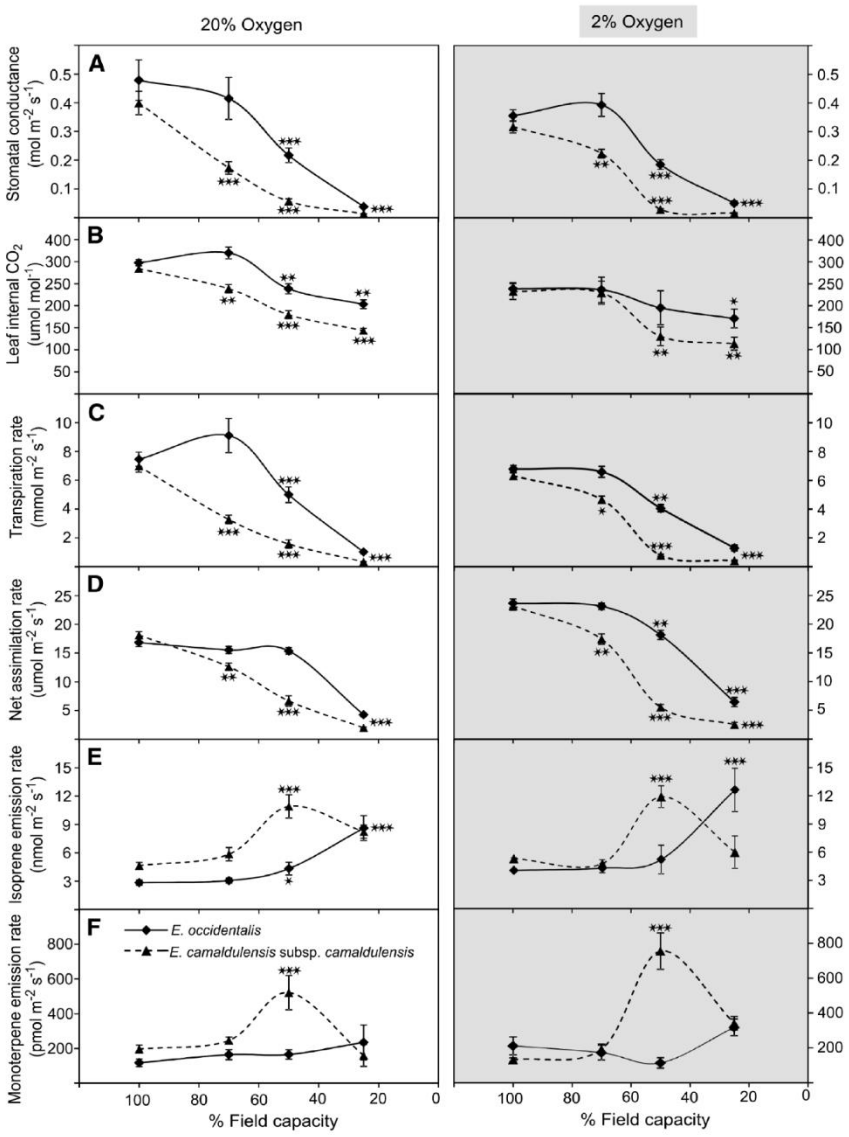

Figure 1. Photosynthesis and isoprenoid emission rates over a drought gradient in two eucalypts, *E. occidentalis* (solid line, diamonds) and *E. camaldulensis* subsp. *camaldulensis* (dotted line, triangles), subjected to 20% O_2 (left) and 2% O_2 (right). A, g_s; B, leaf C_i; C, T_r; D, NAR.; E, I_e; F, constitutive M_e. Each point represents $n = 4$ plants; values are means ± SE. $^*P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, comparison within species relative to 100% FC control. Note that the pronounced decline in NAR with drought in *E. camaldulensis* subsp. *camaldulensis* reflected the fact that its stomata were sensitive to soil water status, a mechanism that presumably achieves a minimum necessary transpiration rate per unit of leaf area during drought stress (White et al., 2000).

Fig. S1). ETR-NAR ratios across drought treatments followed a near-significant quadratic regression with total emission rates ($r^2 = 0.75$, $P = 0.12$; Fig. 2C).

Response to Heat and CO_2 in E. camaldulensis subsp. obtusa

Heat caused a significant decline in photosynthesis under elevated CO_2 (1,000 μmol mol^{-1} or greater; $P < 0.001$). Leaves at 38°C transpired at a significantly higher rate than those at 28°C ($P < 0.0001$), except under drought. Heat did not cause a significant change in either g_s or T_r under normal O_2 in plants under drought (50% FC). This was true across most of the CO_2 range (400–1,800 μmol mol^{-1}), except at 180 μmol mol^{-1} CO_2 and at the photorespiratory compensation point (60 μmol mol^{-1}; normal O_2). Leaves of well-watered plants did not show a decline in NAR when subjected to 38°C under present-day ambient CO_2 (400 μmol mol^{-1}) and normal O_2 (Fig. 3A). Positive effect of heat on T_r was pronounced at $CO_2 \leq 400$ μmol mol^{-1} (Supplemental Fig. S2).

Response to Varying O_2 Concentrations

Net assimilation rate in both species increased significantly (greater than 30%) during low O_2. The gain was proportional to their respective basal rates under normal O_2 except for *E. camaldulensis* subsp. *camaldulensis* at FC ≤ 50% (Fig. 1B; Supplemental Fig. S3). During low O_2,

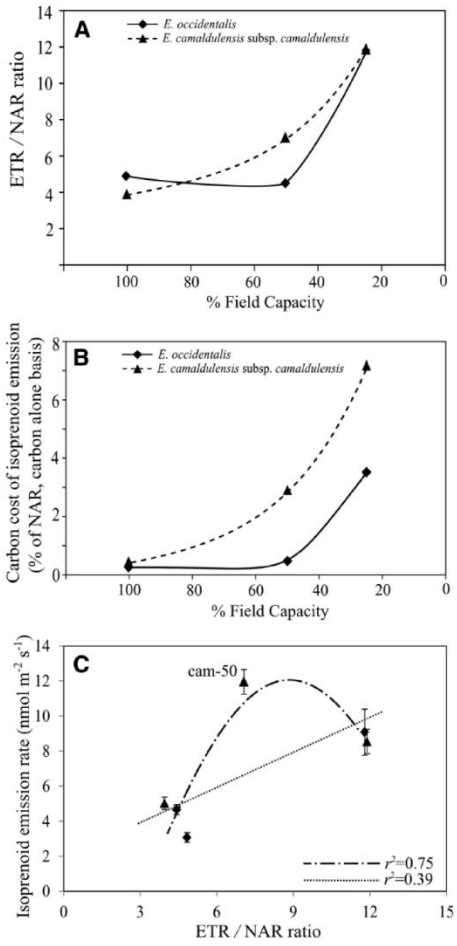

Figure 2. A, ETR-NAR ratio. B, Carbon cost. C, ETR-NAR ratio versus total isoprenoid emission rates at 20% O_2. The relative response of linear ETR and NAR in two eucalypts over a drought gradient is shown. Note the greater decline in NAR in *E. camaldulensis* subsp. *camaldulensis* at 50% FC, which results in a large ETR-NAR ratio. Emission is almost twice as expensive under all conditions on a carbon basis in *E. camaldulensis* (the drought-sensitive species). The regression fits in C encompass data points from both species ($n = 4$; values are means ± SE for emission rates; quadratic $P = 0.12$, linear $P = 0.18$). If *E. occidentalis* were subjected to harsher droughts (FC ≤ 10%), it is also likely to have followed a quadratic regression, with peak emission at 25% FC and declining emission thereafter due to carbon limitation despite a favorable ETR-NAR ratio.

T_r did not change significantly at 100% FC (equal means, $P = 0.002$) despite decreases in g_s and (as a result) a decrease in leaf internal CO_2 (C_i). T_r was insensitive to CO_2 concentrations under low O_2 in *E. camaldulensis* subsp. *obtusa* ($P > 0.1$; Supplemental Fig. S2). Low O_2 had a significant negative effect on transpiration and net assimilation in *E. camaldulensis* subsp. *camaldulensis* only under acute water deficit (FC ≤ 50%, $P < 0.0001$; Fig. 1C; Supplemental Fig. S3). High O_2 (50% O_2) caused a significant increase in C_i and a severe decline in net assimilation rate in *E. camaldulensis* subsp. *camaldulensis*, and the effect was persistent and amplified under drought (Supplemental Table S1).

Volatile Isoprenoid Emission

Response to Drought

Branch-level basal isoprene emission rate (I_e) at 25°C, 1,200 μmol m^{-2} s^{-1} photosynthetically active radiation, and present-day CO_2 and normal O_2 was 40% higher ($P = 0.004$) in *E. camaldulensis* subsp. *camaldulensis* (5.9 ± 1.48 nmol m^{-2} s^{-1}) than in *E. occidentalis* (3.4 ± 1.99 nmol m^{-2} s^{-1}), and the rates remained unchanged in both species despite acclimation to moderate drought (70% FC). The trends were conserved in leaf-level measurements. There was a tiny decrease (relative to control) in NAR in *E. occidentalis* at 50% FC (−1.5 μmol m^{-2} s^{-1}), and it was accompanied by a marginally significant increase in I_e (+1.4 nmol m^{-2} s^{-1}; $P = 0.05$; Fig. 1E [note that there is a 3 orders of magnitude difference between amounts of carbon fixed via photosynthesis and carbon lost via emission]). The biggest increase in I_e for these two species occurred at two different drought intensities. I_e peaked significantly at 50% FC for *E. camaldulensis* ($P < 0.005$; Fig. 1E) and at 25% FC for *E. occidentalis* ($P < 0.001$). These emission peaks coincided with the first noticeable increase in their respective ETR-NAR ratios (Fig. 2, A and C), but the emission declined for *E. camaldulensis* subsp. *camaldulensis* even though ETR-NAR increased further at 25% FC. Constitutive monoterpene emission rate (M_e; pinenes and D-limonene) in *E. camaldulensis* subsp. *camaldulensis* behaved in a manner similar to isoprene, while M_e in *E. occidentalis* did not respond to drought even at 25% FC. I_e and M_e in both *E. occidentalis* and *E. camaldulensis* subsp. *camaldulensis* were comparable in magnitude across the drought gradient (Fig. 1, E and F): the I_e:M_e molar ratio was roughly 10:1 in both species. *E. camaldulensis* subsp. *obtusa* showed a basal I_e:M_e molar ratio of approximately 2:1.

Response to Heat and CO_2 in E. camaldulensis subsp. obtusa

Heat was the most significant factor causing an increase in I_e ($P < 0.0001$) and M_e ($P < 0.001$) across all treatments. At 28°C, 100% FC ($n = 6$), and 20% O_2, I_e showed a significant peak at 180 μmol mol^{-1} CO_2 ($P < 0.001$) and almost full inhibition at the saturating CO_2 level of 1,800 μmol mol^{-1} (Figs. 3B and 4). This response completely disappeared at 38°C (Fig. 3B). Under normal O_2, the I_e response at 50% FC without heat stress (28°C) was equivalent in magnitude to I_e observed in 100% FC plants subjected to heat stress (38°C; $P < 0.001$) irrespective of CO_2 acclimation (Supplemental Fig. S4). Plants acclimated to drought (50% FC) and exposed to heat stress (38°C) showed the highest isoprenoid emission. High-CO_2-induced inhibition of isoprene emission at 28°C disappeared at 38°C and was accompanied

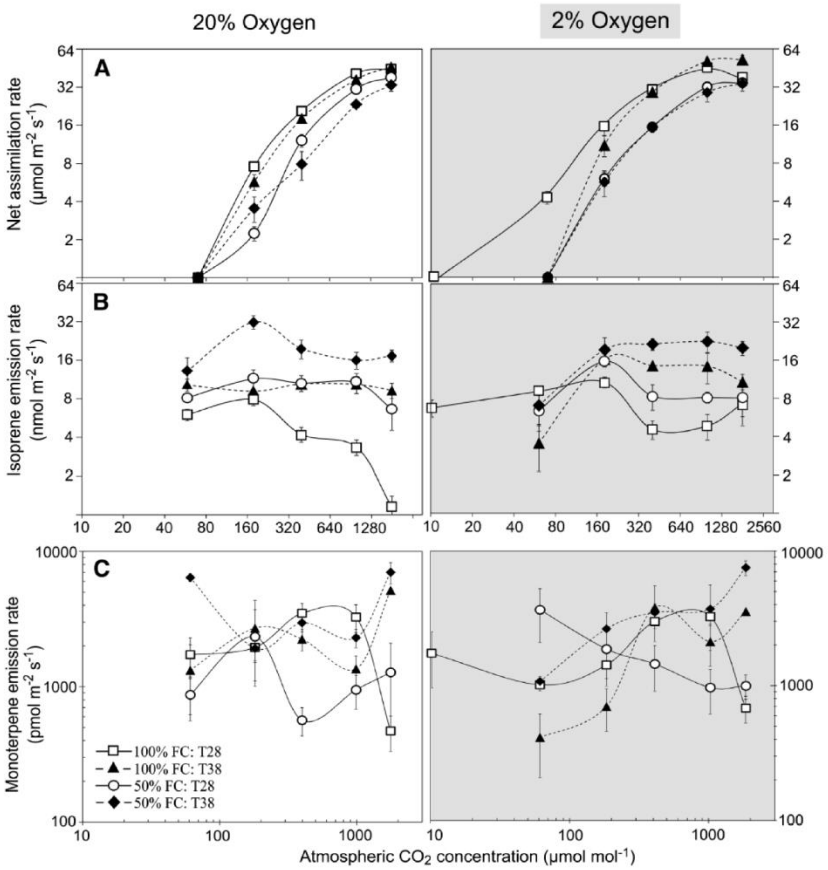

Figure 3. Photosynthesis (A), I_e (B), and constitutive M_e (C) in response to short-term heat stress under 20% O_2 (left) and 2% O_2 (right) over a CO_2 concentration span (60–1,800 μmol mol^{-1}) in *E. camaldulensis* subsp. *obtusa* (experiment 2) acclimated to well-watered conditions (100% FC) and drought (50% FC) at two independent temperature treatments (28°C and 38°C). $n = 6$; values are means ± SE.

by a significantly low g_s and low leaf internal CO_2 (Supplemental Fig. S3).

Response to Varying O_2 Concentrations

Exposure to 2% O_2 (10 min) resulted in a marginal increase in I_e and M_e across the drought gradient (Fig. 1, E and F), and these trends were conserved in both *E. occidentalis* and *E. camaldulensis* subsp. *camaldulensis*, except that the latter showed no significant change in I_e at 100% and 70% FC. Low O_2 on its own did not significantly affect M_e in *E. occidentalis* (Fig. 1F) or in *E. camaldulensis* subsp. *obtusa* ($P > 0.6$; Fig. 3C). Low O_2 significantly increased I_e at CO_2 = 1,800 μmol mol^{-1} (no heat stress) compared with lower CO_2 levels, which was opposite to the effect under normal O_2 ($P < 0.0001$). Low O_2 also had a positive effect on the I_e of well-watered plants at 38°C. In *E. camaldulensis* subsp. *camaldulensis*, I_e increased when well-watered plants were exposed to 50% O_2 (relative to plants at 20% O_2). Emission rate decreased significantly when the plants simultaneously experienced drought (50% FC) and high O_2 (50% O_2). The effect of low O_2 on I_e was more pronounced than on M_e in all three taxa ($P < 0.001$), and increased I_e under low O_2 resulted in a decreased M_e in well-watered plants (Fig. 5, A and C).

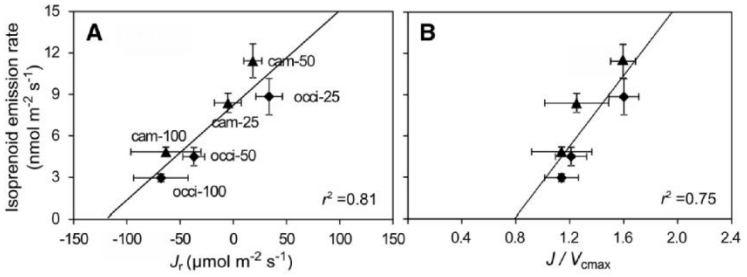

Figure 4. The energetic status model. A, Relationship between residual ETR J_r (not used for light-independent reactions of photosynthesis) and isoprenoid emission rate at different drought stress levels in *E. occidentalis* (black rhomboids) and *E. camaldulensis* subsp. *camaldulensis* (black triangles). B, J/V_{cmax} versus isoprenoid emission rate in both species. $n = 3$ for J_r and J/V_{cmax}, values are means ± SD; $n = 4$ for isoprenoid emission rate, values are means ± SE ($P < 0.05$).

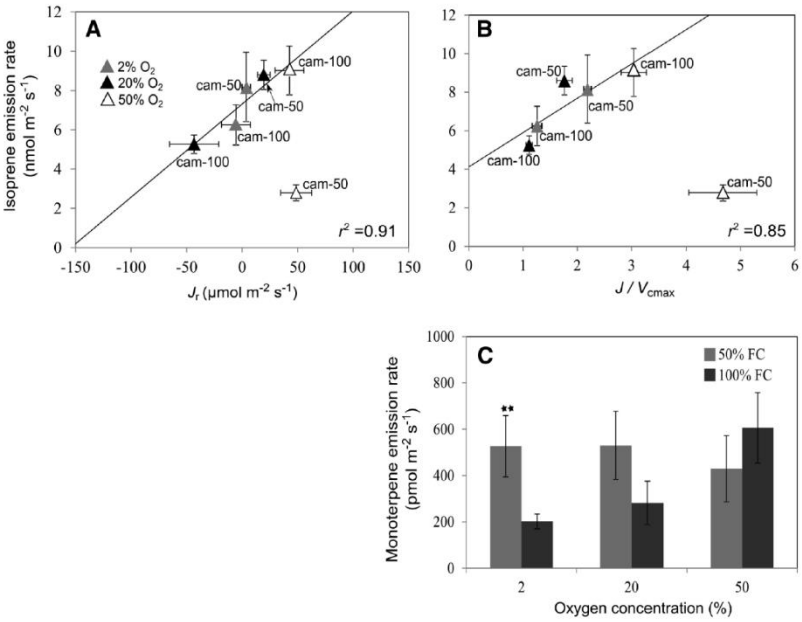

Figure 5. Photorespiration, drought, and isoprenoid emission. A, J_r versus I_e (r^2 = 0.91, P = 0.02) and B, J/V_{cmax} versus I_e (r^2 = 0.85, P = 0.06), in *E. camaldulensis* subsp. *camaldulensis* (experiment 3) acclimated to well-watered (100% FC) and droughted (50% FC) conditions and exposed to three different levels of oxygenated atmospheres. Two percent and 50% O_2 exposure were maintained for 10 to 15 min before volatile sampling (n = 5; values are means ± SE). The correlation remains significant ($P < 0.05$) even when both isoprene and monoterpenes are considered together. C, Response of constitutive M_e to drought and varying O_2 levels. Drought (50% FC) had a significant positive impact on M_e at both 2% and 20% O_2, which was consistent with the isoprene-like response of monoterpenes in *E. camaldulensis* subsp. *camaldulensis* (Fig. 1F). n = 5; values are means ± SE (**$P < 0.05$).

The Relationship between Leaf Energetic Status and Isoprenoid Emission Rate

The estimated ETR not used for light-independent reactions (J_r) correlated significantly and positively with isoprenoid emission rate in both species (r^2 = 0.81, P = 0.014) and was consistent across the drought gradient (Fig. 4A). The positive relationship between electron transport rate (J)/maximum carboxylation rate (V_{cmax}; derived from A-C_i curves) and isoprenoid emission rate (Fig. 4B) was consistent with the ETR-NAR ratio, peaking with increased emission in both species. The relationship between J_r and I_e estimated at three different O_2 concentrations was significantly positive ($r^2 > 0.91$, P = 0.02) for *E. camaldulensis* subsp. *camaldulensis* (Fig. 5A). Plants experiencing drought (50% FC) exposed to 50% O_2 were the only exception to this trend and showed a significant decline in isoprenoid emission despite large J_r (Fig. 5A). *E. camaldulensis* subsp. *camaldulensis* exposed to extreme photorespiratory stress without drought (100% FC, 50% O_2) and drought without extreme photorespiration (50% FC, 20% O_2) resulted in large increases in J/V_{cmax} (Fig. 5B).

DISCUSSION

Drought, ETR-NAR Ratio, and Constitutive Isoprenoid Emission

The insensitivity of ETR to drought in both species in this study confirmed that the photosystems and the electron transport chain are not susceptible to moderate drought stress (Ben et al., 1987) and that the relative decrease in ETR under drought is proportionately less when compared with decrease in CO_2 assimilation (Cornic and Briantais, 1991; Bota et al., 2004). The absolute rates of isoprene and constitutive monoterpene emission did not change provided that the simultaneous assimilation rate of CO_2 remained unchanged.

The Mediterranean species *E. occidentalis* maintained almost an unchanged NAR and I_e even at 50% FC (Fig. 1, D and E). *E. camaldulensis* subsp. *camaldulensis* showed a gradual and more marked decline in NAR with increasing water deficit. ETR-NAR ratio increased significantly at 25% FC for the former and at 50% FC for the latter, the point where the species showed its highest isoprene and monoterpene emission rates (Figs. 1 and 2, A and C). We attribute the increased I_e under drought to the increased ETR-NAR ratio and the increased availability of reducing power to the MEP pathway among other nonphotosynthetic carbon reduction sinks (Fig. 4). Under severe drought (25% FC), despite a favorable ETR status of its leaves, isoprenoid emissions of *E. camaldulensis* subsp. *camaldulensis* declined, suggesting carbon limitation despite 2% O_2 (see decline in NAR at FC ≤ 50%; Supplemental Fig. S3). It was later confirmed that low-O_2 exposure did not significantly affect the ETR-NAR ratio under both well-watered and drought conditions (Supplemental Fig. S1). Similarly, imposing severe photorespiratory stress (50% O_2) on well-watered plants resulted in increased I_e, and the rates plummeted under drought despite a large pool of residual reducing power (Fig. 5). These results suggested that reactions other than photosynthetic carbon reduction (especially photorespiration and the MEP pathway) compete for reducing power not allocated to carbon assimilation reactions under situations of suboptimal carbon assimilation due to abiotic stress. Although eucalypts may have only a

modest capacity for nonphotochemical quenching (NPQ) due to their typically high photosynthetic capacities and acclimation to high-light habitats, the proportion of energy dissipated through NPQ, which is one of the primary mechanisms to mitigate oxidative stress, increased in *E. camaldulensis* subsp. *camaldulensis* as drought intensified (Supplemental Fig. S5). NPQ is likely to be a significant sink for ETR and may also account for reduced emissions under severe stress (Fig. 5A).

Drought, Photorespiration, and Constitutive Isoprenoid Emission

The direction of change in the rates of isoprene emission and photorespiration in response to many environmental factors is the same, despite the absence of a biochemical (carbon-based) link between the two pathways (Monson and Fall, 1989; Hewitt et al., 1990; Loreto and Sharkey, 1990). In this study, net assimilation and I_e increased by a small yet significant extent in well-watered *E. occidentalis* exposed to short-term low O_2 (Fig. 1, D and E; in agreement with Hewitt et al., 1990), and such an increase persisted under drought. The increased de novo carbon pool and decreased competition for ATP and NADPH may explain the small increase in isoprene emission under low O_2 in well-watered plants, given that ATP could be limiting emissions when carbon is plentiful (Loreto and Sharkey, 1993). Although increased isoprene emission in low O_2 under most conditions may be physiologically important, it is not comparable to the large difference in emission between well-watered plants (low emission) and plants experiencing drought (high emission) under 20% O_2 (Fig. 1E). Increased emission under drought is sustained so long as the intensity of drought is within a species-specific tolerance threshold. When such a threshold was exceeded, I_e decreased significantly (at 25% FC for *E. camaldulensis* subsp. *camaldulensis*; Fig. 1E). Since we did not directly estimate photorespiration rates under drought (which are known to increase significantly under abiotic stress), we increased photorespiratory stress in well-watered plants to mimic alternative scenarios. When photorespiratory stress is extreme (50% O_2) and is coupled with reduced carbon assimilation capacity due to drought (50% FC), the MEP pathway suffers a double jeopardy and is likely deprived of both carbon and reducing power (Fig. 5A).

The cost of carbon due to isoprenoid emission increased nearly 15-fold, from 0.46% of freshly fixed carbon in well-watered plants to 7.2% in severely stressed *E. camaldulensis* subsp. *camaldulensis* (Fig. 2B). Although alternative carbon imported from the cytosol avoids the carbon limitation of the MEP pathway under moderate abiotic stress (Funk et al., 2004; Brilli et al., 2007; Trowbridge et al., 2012), under extreme stress carbon could be limiting due to irreparable biochemical impairment of both the photosynthetic carbon reduction cycle and rates of carbon import into plastids. Whether drought just inhibits carbon assimilation rate through stomatal diffusional limitation or there is a clear biochemical down-regulation is highly debatable (Flexas et al., 2004). Under severe drought stress (especially for *E. camaldulensis* subsp. *camaldulensis*), even those limited numbers of leaves that were retained by the plants could not have recovered fully if the plants were rewatered. In such cases, diffusional limitation was likely compounded by an impairment of photosynthetic biochemical machinery. Limited catalytic activity of the MEP pathway, particularly isoprene synthase (Brilli et al., 2007), could have contributed to decreased emissions under extreme stress.

Drought, Low CO_2, Heat, and Constitutive Isoprenoid Emission

In *E. camaldulensis* subsp. *obtusa*, short-term acclimation to heat stress (38°C) caused significantly higher emissions with no significant change in net assimilation despite drought (50% FC; Fig. 3). CO_2 inhibition of isoprene emission also disappeared at high temperatures (for review, see Sharkey and Monson, 2014). It is known that moderate heat stress can suppress ETR yet increase both V_{cmax} and I_e (Dreyer et al., 2001; Darbah et al., 2008). Cold and heat treatments have been shown to selectively suppress PSII (linear electron transport) and thus reduce NADPH availability and up-regulate PSI (cyclic electron transport) to increase ATP production (Huner et al., 1993; Zhang and Sharkey, 2009). All of these observations appear to contradict the view that

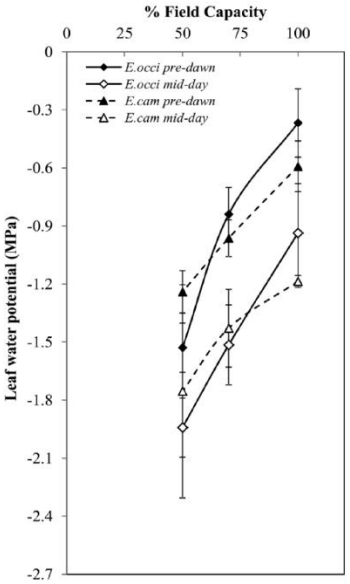

Figure 6. Relationship between percentage FC and leaf water potential in *E. occidentalis* and *E. camaldulensis* subsp. *camaldulensis* (experiment 1).

reducing power availability (however small the requirement may be) influences variation in volatile isoprenoid emission. For the moment, if we ignore cold stress, which is not relevant to isoprene emission, at least to the extent that we understand the phenomenon today, the energetic status model may be inadequate to explain emission behavior at high temperatures for the following reasons: (1) prolonged heat stress reduces net assimilation rate (despite an increase in V_{cmax}), primarily due to decreased CO_2 solubility and decreased Rubisco-CO_2 affinity (Sage and Kubien, 2007); (2) prolonged heat and drought stress (when imposed together) reduce emission, possibly due to heat sensitivity of the cytosolic carbon pool (Fortunati et al., 2008; Centritto et al., 2011); and (3) extreme stress not only reduces net assimilation rates but also increases the photorespiratory drain on carbon and reducing power (Fig. 5).

Unlike isoprene emission, the response of constitutive monoterpene emission did not follow a consistent pattern across any of the treatments (Figs. 1F and 3C). In *E. camaldulensis* subsp. *camaldulensis*, constitutively emitted monoterpenes behaved like isoprene, while in *E. occidentalis*, monoterpene emission was not sensitive even to severe drought. This could be partly due to sustained monoterpene synthase activity during drought, as reported in evergreen oaks (*Quercus* spp.; Lavoir et al., 2009). Oddly, isoprene emission increased in leaves simultaneously exposed to 28°C, 1,800 μmol mol^{-1} CO_2 (not saturating for eucalypts), and 2% O_2 (Fig. 3B). For reasons unknown, the same leaves also showed a significant (16%) decrease in net assimilation rate (Fig. 3A). We speculate that the possible (temporary) inhibition/down-regulation of other sinks of reducing power, such as photoassimilation of N_2 under very high CO_2 (Bloom et al., 2002), also could have contributed to increased isoprene emission. However, it is acknowledged that both the photosynthetic carbon reduction cycle and nitrate assimilation do not compete directly for reducing power (Robinson, 1988). A tradeoff between isoprene and monoterpene emissions in response to CO_2 (28°C; Supplemental Fig. S6) and low O_2 (Fig. 5C) indicates that the emission of isoprene and monoterpenes is inversely related (Harrison et al., 2013).

Increased secondary metabolism and specifically increased isoprene emission under drought could be involved in protecting photosystems against transient periods of oxidative and heat stress, as seen in some transgenic studies, although the mechanisms are unclear (Behnke et al., 2007; Velikova et al., 2011; Selmar and Kleinwächter, 2013; Ryan et al., 2014). However, the tiny increase in isoprene emission when photorespiration (the largest photoprotective sink) is suppressed, despite the down-regulation of photosynthetic carbon reduction under drought, suggests that the MEP pathway has limited capacity to oxidize the pool of excess reductants available under abiotic stress. The increased isoprenoid emission rates among plants experiencing drought (without heat stress), heat (without drought stress; Supplemental Fig. S4), and artificially increased photorespiratory stress (without drought and without heat at 50% O_2) were quantitatively equivalent, and more experiments are needed to differentiate the underlying mechanisms between these responses.

CONCLUSION

The energy (ATP) and reducing power (NADPH) budgets of the chloroplast are used in a hierarchical fashion. The photosynthetic carbon reduction cycle dominates, while, possibly, all other reducing sequences colocalized in the chloroplasts must compete for the remaining pool (Table I). The equilibrium between the source (light reactions) and major sinks (carbon reduction and photorespiration) of energy, as well as the sources (de novo and stored) and sinks (all anabolic processes) of carbon, becomes distorted under drought stress. Drought-induced reduction in the photosynthetic carbon reduction cycle is accompanied by an increase in ETR-NAR ratio and a significant increase in volatile isoprenoid emission. The qualitative response of isoprenoid emission under drought may be similar among species, but the degree of drought-induced shift in J/V_{cmax} is species specific. While energy availability is clearly the common factor that underpins the individual effects of low CO_2 (Morfopoulos et al., 2014), heat, drought, and photorespiratory stress (this study) on isoprenoid emission, the complex interactive effects of heat, CO_2, and drought seem to defy simple assumptions and remain largely uncertain (Fig. 3). Variation in atmospheric O_2 concentration between 10% and 35% during the last 200 million years (Falkowski et al., 2005) and its influence on carboxylation efficiency also could have played an important role in regulating global isoprene emissions on a macroevolutionary time scale. All of these indicate a need for a wider experimental analysis across different functional plant types and ecosystems if we are to reliably scale up volatile isoprenoid emissions from plants to large regions.

MATERIALS AND METHODS

This study included three independent and yet mutually supporting experiments (Table II).

Rationale for Selecting Species

A group of 15 species of eucalypts belonging to distinct biomes within Australia were screened for photosynthetic performance and isoprenoid emission potential in March 2012. The first experiment involved a paired comparison of isoprenoid emission rates and photosynthesis in *Eucalyptus occidentalis* and *Eucalyptus camaldulensis* subsp. *camaldulensis* in response to drought acclimation. *E. camaldulensis* subsp. *camaldulensis* and *E. occidentalis* were studied as a pair in experiment 1, as both had comparable photosynthetic capacities and both predominantly emitted isoprene and some monoterpenes at significant levels. A desirable contrast in the physiology of their water relations is already highlighted (see introduction).

The second experiment tested whether known CO_2 and heat responses of isoprenoid emission are consistent under drought acclimation. The idea was to test any potential pathway discrimination toward either isoprene or monoterpene emission under abiotic stresses. *E. camaldulensis* subsp. *obtusa* was selected for experiment 2 because it emitted comparable quantities of isoprene and constitutive monoterpenes.

The third experiment was a supplementary exercise that was inspired by the results of the first experiment. The third experiment explicitly tested the

relationship between photorespiration and isoprenoid emission under drought in *E. camaldulensis* subsp. *camaldulensis*.

Eucalypts store monoterpenes, and it is difficult to estimate instantaneous carbon and energy invested in monoterpenoid biosynthesis. However, we took necessary precautions to rule out monoterpene emissions from stored pools (from leaf glands). Even if one considers both constitutive and stored monoterpenes together, the quantities are smaller (in the paired species of experiment 1) than that of isoprene emission by 1 order of magnitude. Besides, it was recently shown that stored monoterpenes are quantitatively insensitive to drought stress in eucalypts, although there are clear qualitative variations and inconsistent trends in secondary metabolite accumulation (phenolics and terpenoids) in various plants under drought (Brilli et al., 2013; Selmar and Kleinwächter, 2013).

Plant Material

Seeds of *E. camaldulensis* subsp. *camaldulensis* and *E. occidentalis* were obtained from the Australian Tree Seed Centre at the Commonwealth Scientific and Industrial Research Organization and germinated in May 2012. Two- to 3-month-old seedlings (eight per species) were transplanted to large pots comprising approximately 80 kg of red clay loam (from the Robertson area in New South Wales) and the required quantities of Osmocote slow-release fertilizer. An independent group of *E. camaldulensis* subsp. *obtusa* ($n = 6$) was established in similar large pots. The plants were grown under the open sun with regular watering. Six-month-old saplings (December 2012) were transferred to and kept until the end of the experiment in a glasshouse maintained at a 25°C/18°C diurnal temperature cycle and a natural photoperiodic regime. A third independent group of *E. camaldulensis* subsp. *camaldulensis* ($n = 5$) were germinated in March 2013 and grown in a similar manner for 1 year (used for experiment 3). These plants were maintained at 100% FC, and isoprenoid emission rates were determined at three different O_2 levels (2%, 20%, and 50%) in April and May 2014. After the measurements were complete, the plants were droughted to achieve 50% FC and maintained (over 10 d). Gas-exchange measurements and volatile sampling were repeated.

Water Relations

The soil water-holding capacity was determined by water saturation and weighing. Five-month old saplings were watered and weighed (pot + plant) to obtain the 100% FC reference point. Plants were grouped into two sets of four biological replicates per species. One set was maintained at 100% FC throughout the experiment, while the other received reduced water to achieve 70% FC (within 2 weeks) and was maintained thereafter for 3 months. After volatiles were sampled from plants acclimated to 70% FC, watering was further reduced to achieve 50% FC and acclimated for 2 weeks followed by volatile sampling (repeated for 25% FC). One batch of *E. camaldulensis* subsp. *obtusa* was acclimated to 50% FC for 1 month before volatile sampling. The difference in acclimation period between experiments 2 and 3 is due to the species involved. In experiment 2, we had *E. camaldulensis* subsp. *obtusa*, which comes from lower latitudes of Australia and grows in some of the driest places on the continent. It could endure longer periods of drought and also took longer to acclimate (checked by measuring g_s on randomly selected leaves), while *E. camaldulensis* subsp. *camaldulensis* stabilized more quickly as well as reflected the effects of drought sooner. In experiment 3, we had only *E. camaldulensis*, and we could shorten the acclimation period. The duration of severe stress was shorter than the duration at 70% FC to avoid severe defoliation (especially in *E. camaldulensis* subsp. *camaldulensis*), which would have otherwise hampered emission measurements. Leaf water potential was determined (experiment 1) using a 12-channel thermocouple psychrometer (JRD Merril Specialty Equipment) calibrated at 25°C using standard sodium chloride solutions (Lang, 1967). Leaf discs (diameter = 0.5 cm) were bored out at predawn from half of a fully expanded leaf and transferred and sealed in the psychrometer (10 leaves per treatment group). The chambers were equilibrated in a water bath at 25°C for 3 h before signal acquisition. The procedure was repeated on the following midday using a leaf disc punched out from the other half of the same leaf. Measurements were repeated at three time points during drought treatment (for the relationship between leaf water potential and percentage field capacity for a physiological assessment of drought intensity, see Fig. 6).

Monitoring Photosynthesis under Drought

Photosynthetic gas-exchange measurements were made using a LI-6400XT (Li-Cor Biosciences) infrared gas analyzer. During branch-level sampling, photosynthesis was measured between 12 noon and 3 PM in parallel to isoprenoid sampling on independent branches of the same plant. Leaf temperature was 25°C, light intensity was 1,200 μmol m^{-2} s^{-1}, and relative humidity ranged from 39% to 47%.

A-C_i curves were obtained from one or two of the best performing healthiest leaves from each biological replicate ($n = 4$) per treatment group using a Li-Cor6400 at 25°C and normal O_2 (also at 2% and 50% O_2 for experiment 3). V_{cmax} and J were estimated using the curve-fitting tool described by Sharkey et al. (2007; minimized errors), and the mean values were used as model input parameters. J_r (given C_i and V_{cmax}) was calculated following Harrison et al. (2013) and Morfopoulos et al. (2014):

$$J_v = 4V_{cmax}\left[\frac{C_i + 2\Gamma^*}{C_i + K_m}\right] \quad (1)$$

$$J_r = (J - J_v) \quad (2)$$

Given V_{cmax}, we calculated J_v and then substituted Equation 1 in Equation 2, where J_v = proportion of electron transport used for dark reactions, V_{cmax} = maximum carboxylation rate by Rubisco, K_m = effective Michaelis-Menten coefficient for carboxylation by Rubisco (700 μmol mol^{-1} at 25°C), Γ^* = photorespiratory compensation point (60 μmol mol^{-1}), and C_i = species-specific leaf internal CO_2 concentration (μmol mol^{-1}) at ambient CO_2 = 400 μmol mol^{-1}.

Chlorophyll fluorescence was monitored using a Pocket PAM chlorophyll fluorescence meter (Gademann Instruments). Kautsky dark-light fluorescence induction curves were obtained from dark-adapted leaves (predawn) between 3 and 5 AM and replicated on 15 fully expanded leaves per treatment group ($n = 4$; biological replicates). The measurements were made in a dark room (photosynthetic photon flux density < 10 μmol m^{-2} s^{-1}). Predawn leaf temperature was 15.5°C ± 0.3°C. During the day (between 2 and 4 PM), leaves experiencing moderate light levels (350 $<$ photosynthetic photon flux density < 450 μmol m^{-2} s^{-1}) were used to estimate linear ETRs using steady-state light induction curves by gradually increasing the pulse intensity from 0 to 1,500 μmol m^{-2} s^{-1}. Predusk leaf temperature was 24.5°C ± 0.8°C. The data for ETR-NAR ratio were measured only after the dry group had been acclimated to 50% FC, and the control group (100% FC plants) was retained throughout the experiment.

Volatile Isoprenoid Sampling

Branch Enclosure Method

Tedlar bags (25-L capacity; Sigma) were modified to make a volatile collection chamber with a polytetrafluoroethylene base for air-tight sealing. The gas-exchange line was plumbed with Teflon tubing and stainless steel air-tight connectors (Swagelok). High-purity instrument-grade air (BOC; 78% N_2, 21% O_2, and 1% argon) was mixed with CO_2 (β-mix 5% ± 0.1% in N_2) to achieve ambient CO_2 (400 ± 10 μmol mol^{-1}) concentration in the headspace containing the branch. The unit was flushed at 20 L min^{-1} for 10 min before each sampling to remove memory effects (Niinemets et al., 2011). A branch was inserted into the chamber and sealed around at the base. Plants were provided with natural photosynthetically active radiation at 800 to 1,200 μmol m^{-2} s^{-1} during sampling. The chamber temperature was maintained at 30°C ± 2°C, and leaf temperature was 26°C ± 2°C measured using an infrared thermometer (Agri-Therm III; Everest Interscience). Relative humidity varied from 29% to 52%. Sterile fritted glass thermal desorption (TD) tubes comprising Carboxen 1016 and Carbopack X adsorbents (Sigma Supelco) were conditioned at 250°C through helium purging (100 mL min^{-1}, 60 min). Volatiles were collected into TD tubes in April and May 2013 using an oil-free pump connected to a mass flow controller (Brooks 5850E). Chamber blank control, TD tube secondary desorption control, and branch memory effect (preflush blank) screenings were also performed.

Cuvette-Based Leaf-Level Sampling

An LI-6400XT portable gas-exchange system was suitably modified to sample volatiles directly from the leaf cuvette onto the thermal adsorbent bed described above. The LiCor was supplied with volatile free humidified air mixed with CO_2. Within-cuvette ambient CO_2 was 400 μmol mol^{-1}, leaf temperature was 25°C, humidity ranged from 40% to 62%, and the light intensity was 1,200 μmol m^{-2} s^{-1}. Isoprenoids were also sampled in a low-O_2 (2%), ambient-CO_2 (400 μmol mol^{-1}) atmosphere generated by mixing N_2, high-purity O_2, and CO_2 at the required ratio and humidified to achieve 40% to 50% relative humidity in the cuvette. Leaves were exposed to low O_2 or

high O_2 for 5 to 10 min (stable readings) prior to sampling. The effect of varying O_2 exposure on photosynthesis was also studied independently (although simultaneously) on many leaves.

Sampling from E. camaldulensis subsp. obtusa under Controlled CO_2, O_2, Temperature, and Water Availability

One-year-old saplings (n = 6) were maintained at 100% FC (volatiles were sampled), then gradually dried down to 50% FC, and again acclimated for 15 d (volatiles were sampled again). Before sampling, individual leaves were exposed for 10 min to five possible atmospheric CO_2 concentrations (60, 180, 400, 1,000, and 1,800 μmol mol^{-1}), two temperatures (28°C and 38°C), two O_2 concentrations (20% and 2%), and saturating light intensity (1,500 μmol m^{-2} s^{-1}). The CO_2 compensation point remained close to 60 μmol mol^{-1} in all treatments except in well-watered plants at 28°C (low O_2), where it was 10 μmol mol^{-1}. CO_2 treatment was randomized so that on a given day, some leaves from different biological replicates received low-CO_2 treatment while others received high-CO_2 treatment to avoid either stimulation or limitation of photosynthesis.

Thermal Desorption Gas Chromatography-Mass Spectrometry Analysis

A Shimadzu GCMS-QP 2010 fitted with an auto thermal desorption system (TD-20) was used for offline volatile analysis. Ultrapure helium (BOC) was used as the carrier gas. Isoprene and D-limonene (analytical grade; Sigma) were injected into sterile 5-L Tedlar bags comprising N_2 to generate standard mixing ratios. Then, the standard mixture was adsorbed onto TD tubes (described above), which were used to calibrate the instrument at regular intervals. Isoprene sampled from the plant chamber was desorbed from the TD tube at 220°C (60 mL min^{-1} for 5 min). A 30-m, 25-mm i.d., 25-μm RTX-5 Sil MS (Restek) capillary column was used for gas chromatography. The temperature regime for the gas chromatography run was 28°C (3 min) to 110°C (3 min) at 5°C min^{-1} and finally to 180°C at 5°C min^{-1}. The chromatographic peaks were identified by comparing them with isoprene and monoterpene standards (α-pinene and D-limonene) and reference mass spectrographs in the National Institute of Standards and Technology Standard Reference Database 1A (National Institute of Standards and Technology, 2008). I_e was calculated by utilizing sampling flow rate, total leaf area in the sampling chamber (for branches), and quantified isoprene standards.

Statistical Analysis

The statistical tests were performed using Minitab (version 16 statistical package). The equality of means in responses within species between two treatments was analyzed using paired Student's t tests. Differences in mean responses between two species to the same treatment were subjected to two-sample Student's t tests. The CO_2, O_2, temperature, and drought intensity interactions were analyzed using a multilevel general full factorial model with ANOVA (Montgomery, 2004). Experiments had four (between species) to six (sequential) biological replicates and eight to 15 independent leaf-level measurements (technical replicates) per treatment group.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Electron transport rate to net assimilation rate ratio in response to varying levels of oxygen concentration in *E. camaldulensis* subsp. *camaldulensis*.

Supplemental Figure S2. Photosynthesis response to short-term heat stress.

Supplemental Figure S3. Short-term response of photosynthesis to low O_2.

Supplemental Figure S4. Comparison of emissions under heat without drought and drought without heat.

Supplemental Figure S5. Estimated NPQ across drought gradient.

Supplemental Figure S6. Isoprene and monoterpene emission rates peaking at two different CO_2 concentrations.

Supplemental Table S1. Photosynthesis parameters of *E. camaldulensis* subsp. *camaldulensis* in response to drought and photorespiratory stress.

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Chapter 4

The curious case of *post-illumination monoterpene burst* [4]

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Manuscript in preparation

[4] **Contributions**: KGSD developed the hypothesis that a potential link between post-illumination CO_2 burst may regulate post-illumination isoprene/monoterpene bursts. Experiments were designed and developed by KGSD with expert inputs from FL, MC and GM (all at CNR, Italy). PTR-MS measurements were made by KGSD and GM with the help of researchers at University of Firenze. A report comprising results presented in this chapter was submitted by KGSD of Macquarie University (Sydney, Australia), towards the partial fulfilment of a research grant awarded to him by Consiglio Nazionale delle Recerche in Italy under its STM Scheme-2013.

Abstract:

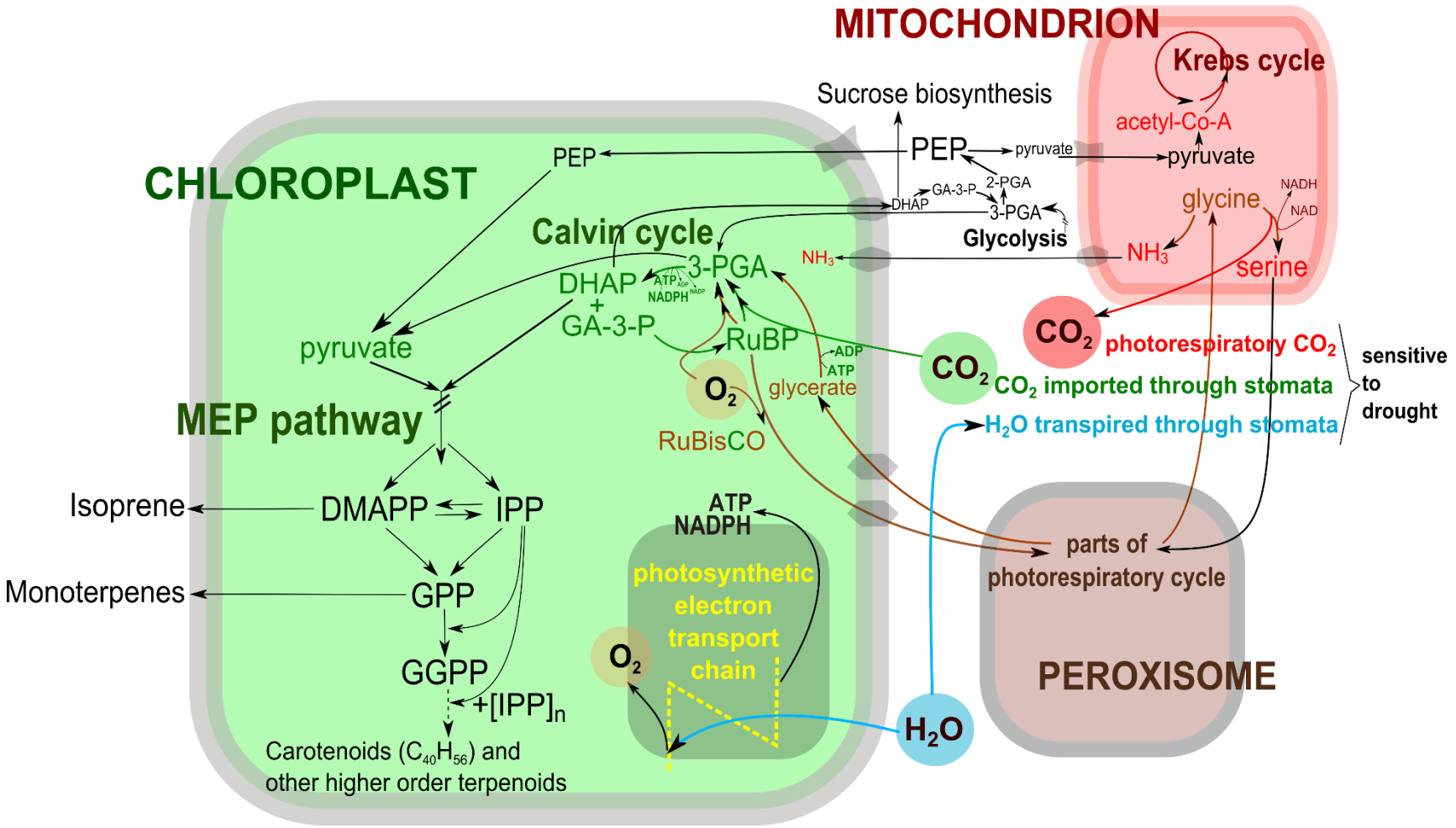
Light-dependent processes localized in plant chloroplasts cease immediately when subjected to darkness, but shortly after darkness is imposed (*post-illumination*), some of these processes show transient activity. The study of such processes has in the past provided insight into the relationship between colocalised light-dependent pathways in the chloroplast. *Post-illumination isoprene burst* (*PiIB*) is one such process and its intensity is determined by light and temperature. We intended to test whether regulation of constitutively emitted non-stored monoterpenes is similar to that of isoprene. We employed monoterpene emitting *Quercus ilex* (holm oak), an evergreen oak from the European mediterranean biome, and *Eucalyptus camaldulensis* to explore the occurrence and regulation of *post-illumination monoterpene burst* (*PiMB*). In our experiments, we saw *PiMB* to be an inconsistent and rare phenomenon occurring in some leaves and absent in most within the same plant of *Q. ilex*. We also found that post-illumination CO_2 burst, a phenomenon far more common among C_3 plants including *E. camaldulensis*, was absent in *Q.ilex*. We observed that monoterpenes were preferred to isoprene in *E. camaldulensis* in post-illumination burst. We discuss these results along with other important findings in the context of the role played by photorespiration in post-illumination energy demands within chloroplasts, in addition to substrate affinity of isoprenoid synthases in oaks and eucalypts.

Introduction:

Isoprene (C_5H_8) and monoterpenes ($C_{10}H_{16}$) are volatile hydrocarbons synthesized by the MEP pathway localized in plant chloroplasts. These compounds are emitted in large quantities (1000 Tg C/yr) by plants and they play an important role in the global carbon cycle. Volatile isoprenoids, especially isoprene and monoterpenes have significant impact on NO_x and methane chemistry in the troposphere. Isoprene emission from plants is dependent on light and is influenced by temperature, CO_2 concentration and plant water status. Monoterpenes are synthesized and constitutively emitted in the same way as isoprene in some plants (e.g. *Quercus ilex*). Many plants (e.g. *Eucalyptus* spp) also store monoterpenes that are emitted in a light-independent manner. The interactive effects of CO_2 and heat on photosynthesis and isoprene emission are complex (e.g. Centritto et al. 2004, 2011). Experiments on poplar mutants and transgenic tobacco have provided support to the hypotheses that heat and/or oxidative stress tolerance in plants can be mediated by

isoprene emission (e.g. Behnke et al. 2007; Vickers et al. 2011). In a recent review, we identified major gaps in the current understanding of isoprenoid biology and climate interactions and laid down a direction for future isoprenoid research (Harrison et al. 2013). Taking those perspectives a step forward, we explored many untested physiological aspects of plant isoprenoid emission by using the state-of-the-art facility at the National Research Council (Italy) and University of Florence.

There are several light-dependent processes localized in plant chloroplasts that cease immediately when subjected to darkness. Photorespiration is one such pathway that occurs in three different plant organelles (Fig. on page 74). Constitutive isoprenoid emission (via the MEP pathway) is another example. However, shortly after darkness is imposed (*post-illumination*), some of these processes show transient activity. In pioneering studies Decker (1955) and Tregunna et al (1961) showed that plants exhibit a post-illumination CO_2 burst (*PiCO₂B*) which was sensitive to light intensity acclimation and temperature; it was later shown to be of photorespiratory origin (Ludwig and Krutkov 1964; Ludwig and Canvin, 1971, Tarlowski et al 1986). Similarly, constitutive isoprene emission ceases almost immediately when plants are shifted from light to darkness (e.g. Seemann et al. 2006). However, a short period after exposure to dark plants show transient dark emission of isoprene before it completely ceases again (Monson et al. 1991; Rasulov et al. 2009a; Li et al. 2011). The intensity of post-illumination isoprene burst (*PiIB*), as it is called, is shown to be dependent on the preceding light intensity and temperature acclimation (Rasulov et al. 2009; Li et al. 2011). Both *PiIB* and *PiCO₂B* have two phases in darkness. They both show a large primary peak soon after the light is turned off and primary peak falls quickly to zero, although there are significant differences in duration of *PiIB* and *PiCO₂B*. The secondary CO_2 burst merges with the stabilizing phase of dark respiration (sustained all the time). The secondary burst in isoprene disappears eventually.


MITOCHONDRION
Krebs cycle
acetyl-Co-A
pyruvate
glycine
NADH
NAD
serine
NH3
Sucrose biosynthesis
PEP
pyruvate
GA-3-P
2-PGA
DHAP
3-PGA
Glycolysis
CHLOROPLAST
Calvin cycle
3-PGA
ATP
ADP
NADPH
NADP
DHAP
+
GA-3-P
RuBP
ADP
ATP
glycerate
O2
RuBisCO
pyruvate
MEP pathway
Isoprene
DMAPP
IPP
Monoterpenes
GPP
GGPP
+[IPP]n
Carotenoids (C40H56) and
other higher order terpenoids
ATP
NADPH
photosynthetic
electron
transport
chain
O2
H2O
CO2
photorespiratory CO2
CO2 imported through stomata
H2O transpired through stomata
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to
drought
parts of
photorespiratory cycle
PEROXISOME

The following experiments were designed to test whether regulation of constitutively emitted non-stored monoterpenes is similar to that of isoprene. We employed *Quercus ilex* (holm oak), an evergreen oak from the European mediterranean biome. *Q.ilex* does not emit isoprene rather it emits monoterpenes in a manner similar to isoprene. We explored the occurrence and regulation of post-illumination monoterpene burst (*PiMB*) in *Q. ilex.* We also used *Eucalyptus camaldulensis* and *Populus nigra* as isoprene-emitting system controls. *E. camaldulensis* was also used to test the post-illumination relationship between isoprene and monoterpenes since both come from the MEP pathway.

Materials and methods:

Plant material: Two-year-old potted saplings of *Populus nigra, Eucalyptus camaldulensis*, and *Quercus ilex* were regularly pruned and maintained under natural photoperiod and outdoor conditions. They were initially grown in 5 kg pots and later transplanted to 10 kg pots comprising potting mix, sand and slow release fertilizer. *P. nigra* was given additional artificial light using a fluorescent light bank to avoid leaf-shedding and down-regulation of metabolism in winter.

Photosynthetic performance: A LiCor 6400 (LI-COR Biosciences Inc., PA-USA) portable infra-red gas analyser was used to monitor the physiological status of the plant material. The Peltier cooling system of the leaf cuvette was supplemented with an external circulating water jacket connected to a water bath (Thermo Fisher, Germany) so that the set leaf temperature did not fluctuate >0.3 ˚C during quick light-dark switch over. PAR

intensity was 1500 µmol m^{-2} s^{-1}. Measurements were carried out in triplicate between 10 am and 3 pm during November 2013. Post-illumination CO_2 burst was measured continuously on the same leaf before and after monitoring isoprene/monoterpene bursts. All measurements were repeated at a range of oxygen concentrations (ranging from 2% to 80%).

Monitoring volatile isoprenoid emission using PTR-TOF-MS: Emission measurements were carried out using a customized LiCor6400 fed with VOC-free air mixed with CO_2 into the leaf cuvette. Ultrapure nitrogen, oxygen, and CO_2 (10% in N_2) were mixed using an automated mass-flow controlling circuit (Brooks Instrument, PA, USA). The air was humidified to achieve 40 to 60% RH. Before each measurement, the LiCor cuvette was cleaned with VOC free air and then the leaf was inserted and acclimated to 1500 µmol m^{-2} s^{-1} of PAR, 425±10 ppm of CO_2 and the desired leaf temperature for at least 20 minutes. Isoprene (protonated mass=69) emission was measured continuously using the Proton Transfer Reaction Time of Flight Mass Spectrometer (PTR-TOF-MS, Ionicon Analytik, Innsbruck, Austria). Isoprenoid emission rate was not quantified since the aim initially was to detect consistent occurrence of post-illumination bursts. Leaves of *P. nigra* (at 35 ˚C) were used to test the protocol to monitor post-illumination isoprene burst (modified from Rasulov et al 2009a) including a cuvette cleaning procedure (Li et al. 2011). Protonated fractions of monoterpenes (m/z= 81 and 137) were used to monitor monoterpenes from *Q. ilex* at three different temperatures (25 ˚C, 30˚C, and 35 ˚C) with identical light treatments. Similarly, both isoprene and monoterpenes were tracked in low emitting *E. camaldulensis* at 40 ˚C to get a strong basal signal before light was switched off.

Results and Discussion:

Experiments on the mediterranean evergreen oak *Quercus ilex* (CNR, unpublished) could not confirm the occurrence of post-illumination monoterpene burst (*PiMB*). In our experiments, we saw *PiMB* to be an inconsistent and rare phenomenon occurring in some leaves and absent in most within the same plant (Figure 1; 2 out of 10 leaves on an average showed *PiMB*). We also found that post-illumination CO_2 burst, a phenomenon far more common among C_3 plants (e.g. Zelitch, 1971), was absent in *Q.ilex* (Figure 2). Under low O_2, post-illumination net assimilation rate decreased at a significantly slower rate in *Q. ilex* despite its relatively lower A_{sat} (takes >50s, Figure 2) than in *Eucalyptus camaldulensis* (tended to zero in 20s after light was turned off).

We observed that most leaves, irrespective of whether they exhibited *PiMB* in *Q.ilex* also exhibited *PiCO₂B* with a distinct and transient photorespiratory peak (Figure 3). However, such a peak was consistent only when subjected to hyperoxic conditions ([O_2] >50%). Based on these and other observations (to be discussed), we consider two possible explanations for the erratic behaviour of *PiMB* in *Q. ilex*: (a) They have very low photorespiration costs compared to other plants thus carboxylation is highly efficient (b) They have higher than average photorespiration to cope with hot and dry mediterranean climate and they have evolved a more efficient mechanism to recapture photorespiratory CO_2 as in some crop plants (Loreto et al. 1999; Busch et al. 2013). Given the role played by photosynthetic electron transport rate and the demand for reducing equivalents amongst various pathways within chloroplasts in determining isoprene and monoterpene emission under both drought and stress-free conditions (Niinemets et al. 2002; Dani et al. 2014), the extent to which post-illumination carboxylation continues may give us a handle on post-illumination monoterpene burst in *Q.ilex*.

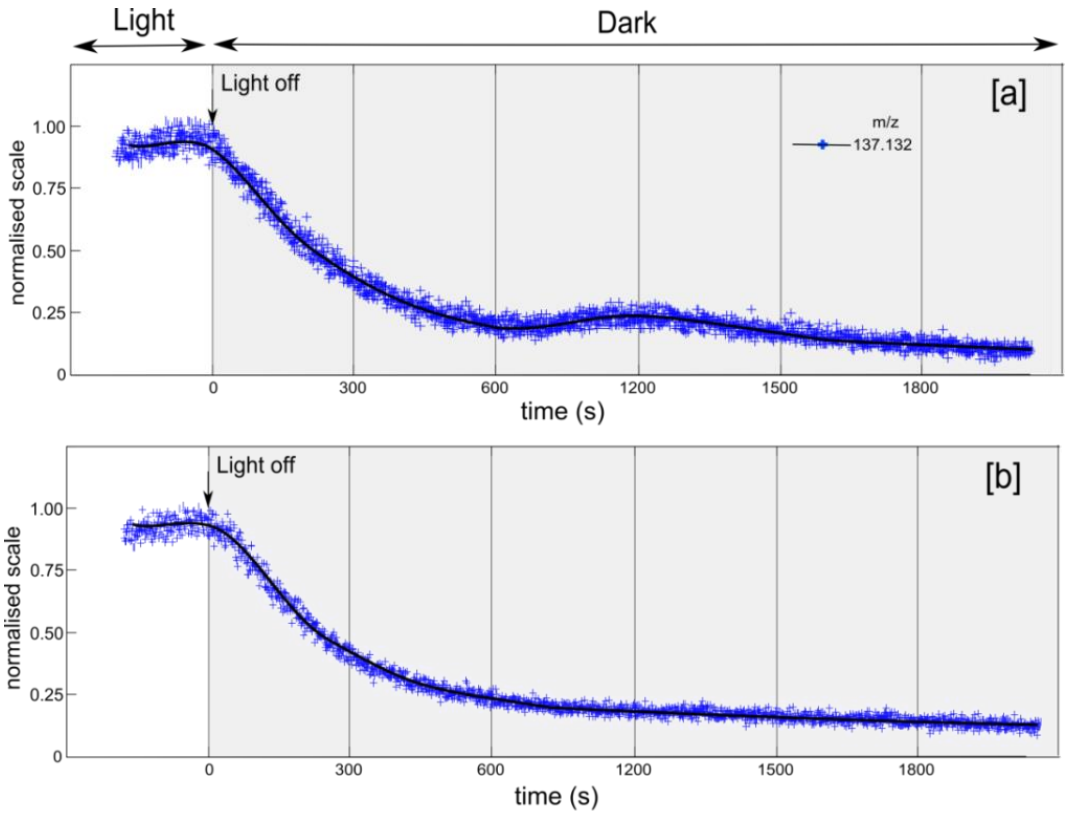

Figure 1: **Inconsistency of post-illumination monoterpene burst in *Quercus ilex*:** [a] an atypical response of constitutive monoterpene emission with a clear post-illumination burst at 1200s post darkness [b] Representative common response where post-illumination monoterpene burst is not observed. Both these real time PTR-MS spectra were obtained under identical conditions from two different leaves. The conditions were 25 $^{\circ}$C, 20 minute acclimation to 1500 µmol $m^{-2}s^{-1}$ of PAR before light was turned off, 425 ± 10ppm CO_2, and 40% RH.

There could be leaf to leaf variation in monoterpene emission rates due to variations in metabolic functional requirements, but it is puzzling that *PiMB* is rare given monoterpenes are always emitted in light by all the leaves in *Q. ilex* (Figure 1). The magnitude of secondary dark-phase peak in *PiIB* is shown to decline under hypoxia despite no change in dark respiration (Rasulov et al. 2011). In contrast, it was shown very early (Tregunna et al. 1966) that the secondary CO_2 burst during *PiCO_2B* is not sensitive to hypoxia and it is largely from dark-respiration, although the reason for a transient and delayed increase in CO_2 output are unknown. If one accepts that depending on leaf ontogeny, dark respiration could compete with isoprene emission for the same substrates from the cytoplasm

(Rosenstiel et al. 2003; Loreto et al. 2007), dissecting the nature and magnitude of a secondary post-illumination CO_2 peak in *Q. ilex* (Figure 2) may hold the key to understanding *PiMB.*

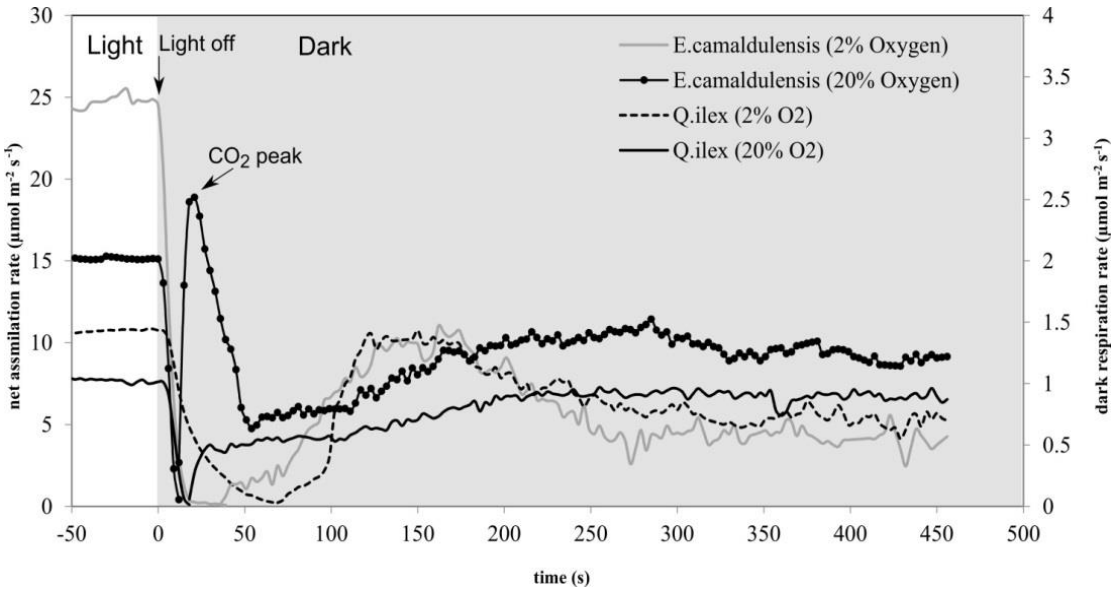

Figure 2: Post-illumination CO_2 burst in *Eucalyptus camaldulensis* and *Quercus ilex*: Under normoxia (20% O_2), the primary CO_2 peak of photorespiratory origin is very clear in *Eucalyptus camaldulensis* and the same is absent in *Quercus ilex.* Both species show a clear slow rising post-illumination CO_2 peak (~150 s post darkness) when acclimated to hypoxic (2%) atmosphere. Reasons for the latter are unknown. Secondary y-axis should be used for data points after the light-off arrow (N = 5)

Isoprene emission is suggested to be regulated by conditions of substrate (DMAPP) limitation and isoprene synthase kinetics (Magel et al. 2006; Rasulov et al. 2010) and DMAPP pools deplete quickly into darkness (Rasulov et al. 2013). A trade-off between carotenoid biosynthesis and isoprene emission (both are products of the MEP pathway) was proposed (Own and Penuelas, 2005) and recently deduced using *PiIB* based DMAPP pool size estimation in the leaves of *Populus tremula* at different stages of leaf development (Rasulov et al. 2014). *E. camaldulensis* confirmed the proposed biochemical trade-off between isoprene and monoterpene emission among emitting genera (Figure 4; Harrison et al. 2013). After turning off the light source (even at 40 °C), isoprene emission declined

and monoterpene emission instantly peaked. Those monoterpenes were unlikely to have come from stored pools, as they were not light-independent.

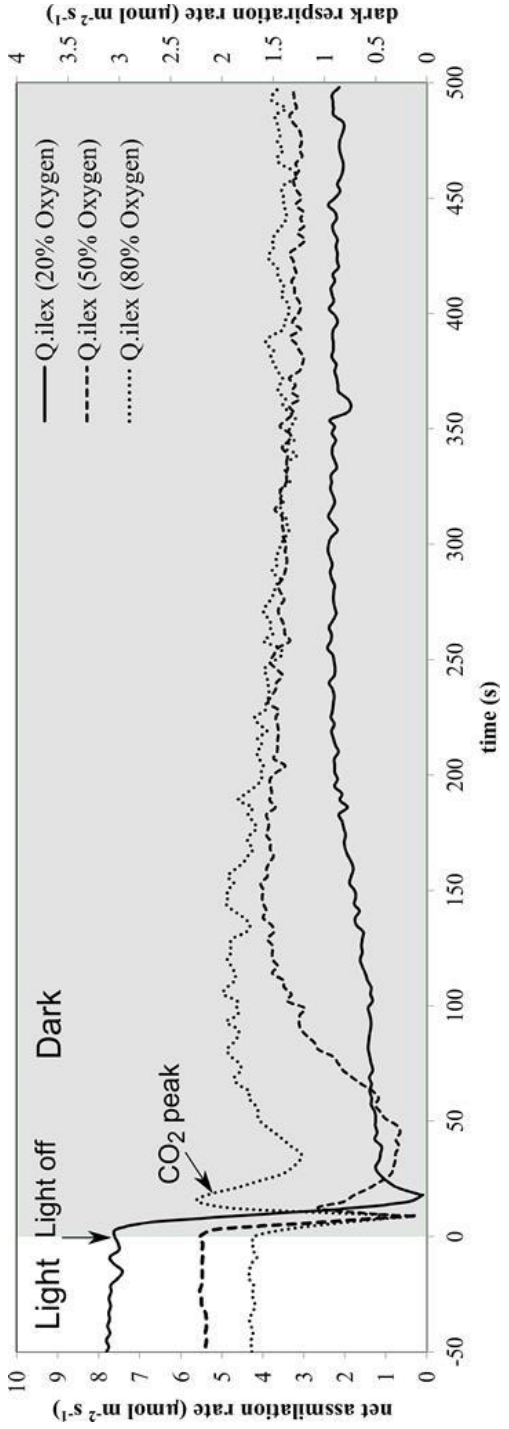

Figure 3: Post-illumination photorespiratory CO_2 burst in *Q.ilex* subjected to hyperoxia: The graph shows that the CO_2 burst which is not seen under normoxia (*N*=7, 20% O_2) starts appearing at 50% O_2 (*N*=3) and becomes more prominent under hyperoxia (*N* = 3, 80% O_2). Secondary y-axis should be used for data points that succeed the 'light-off' arrow.

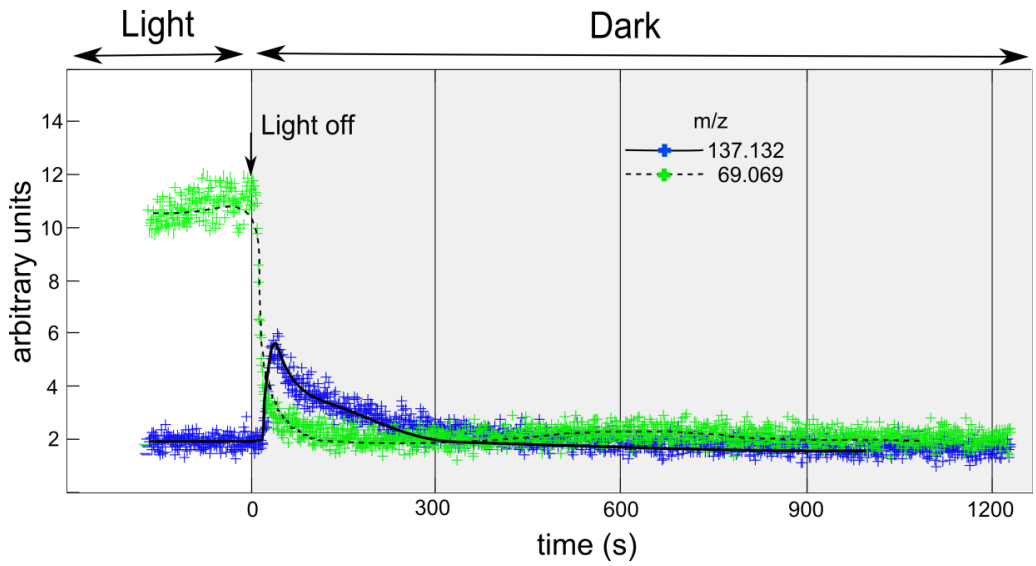

Figure 4: **Post-illumination trade-off between isoprene and monoterpene emission in *Eucalyptus camaldulensis***. Monoterpene emission increases immediately after the light is turned off and the increase coincides with the decline in isoprene emission. Isoprene (m/z =69.069) shows a clear secondary post-illumination burst at 600s, while monoterpenes (m/z =137.132) do not show a clear secondary burst. Leaf was acclimated to 40 $^{\circ}$C, 20 minute acclimation to 1500 µmol $m^{-2}s^{-1}$ of PAR before light was turned off, 410±10ppm CO_2, and 60% RH (N = 3).

Gernyl pyro phosphate (GPP) is f the substrate for monoterpene biosynthesis and GPP is formed by the fusion of IPP and DMAPP. GPP is also first substrate in the sequence of enzymatic reactions leading to the formation of carotenoids. Let us assume that constitutive (non-stored) monoterpene emission is also likely to follow post-illumination isoprene burst-like kinetics. Then we could accept that due to low $K_{m,\ DMAPP,\ IPP}$ (0.01 mM) of GPP synthase, the post illumination increase in monoterpene emission in *E. camaldulensis* continued for 300 s (Figure 4) despite a decreased DMAPP pool size. However, we saw a clear secondary isoprene burst in *E. camaldulensis* while a secondary monoterpene burst was absent. The low rate of monoterpene emission in *E. camaldulensis* when light-driven isoprene emission is high, points towards a limited GPP pool and continued conversion of IPP + DMAPP → GPP (for >300 seconds) even as isoprene emission declined in darkness.

It is proposed that the delayed secondary post-illumination isoprene peak is limited by carbon (*de novo*) trapped as MEP pathway metabolites, which are converted to DMAPP upon a slow and transient supply of reducing power (Rasulov et al. 2011; Li and Sharkey, 2013). Could one conclude that a delayed conversion of MEP pathway precursor to DMAPP leads to a large enough DMAPP pool size that again favours isoprene synthase over GPP synthase? All of these raise questions about substrate-limitation hypothesis explaining post-illumination kinetics of isoprene and monoterpene emission. Perhaps, erratic behaviour of *PiMB* in *Q.ilex* (this study) and the general non-correlation between MEP pathway substrate-pool sizes and isoprenoid emission capacity in plants (Nogues et al. 2006) suggests that monoterpene emission could be sensitive to other extra-chloroplastic factors including respiratory carbon. It remains to be tested whether post-illumination consumption of DMAPP for monoterpene emission in *Eucalyptus* is significant enough to cause complete depletion of MEP pathway intermediates "stuck" due to reducing power limitation.

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Chapter 5

Seasonal rhythm of volatile isoprenoid emission in eucalypts is entrained by temperature and is photoperiod-gated[5]

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Manuscript in preparation

[5] **Contributions**: The hypothesis proposed and tested in this chapter was independently proposed by KGSD and tested by him through experiments that had important designing inputs from BJA. The branch-level measurements were done with technical advice from IMJ. KGSD analyzed the data and wrote the manuscript with editorial inputs from BJA, IMJ and ICP. Roger Hiller and Malcolm Possell acted as internal referees, who are acknowledged.

Abstract:
Light is the *sine qua non* of plant isoprene emission while rates of emission respond strongly to increasing temperatures (Q_{10} >5). Diurnal variation in foliar volatile isoprenoid emission in plants involves highly synchronized transcriptional regulation of the genes involved in the MEP pathway, suggesting broader regulatory implications at the transcriptional level for seasonal variation in emission from perennial plants. We tested the hypothesis that photoperiodic acclimation and transcriptional regulation of the MEP pathway plays a role independent of temperature in influencing seasonal variation in emission. We used seedlings of *Eucalyptus globulus* and *E. camaldulensis* acclimated to two distinct temperatures (25˚C, 33˚C) and photoperiodic regimes (natural day-length and long-day) and monitored photosynthesis, isoprenoid emission rate and abundance of selected mRNA transcript levels. Net assimilation rates did not differ significantly between and across treatment groups, although there were notable variations in relative transcript abundance of selected core photosynthesis genes. Long-day alone caused increased isoprene emission in *E. camaldulensis* while warmer temperature acclimation alone caused increased emission in both the species, all characterised by an associated increase in the transcript abundance of *DXS* (1-deoxy-D-xylulose-5-phosphate synthase) and *ISPS* (isoprene synthase). Diurnal oscillation in volatile emission is temperature compensated whilst the phase-width is determined by photoperiod. We propose that seasonal maxima and minima in plant volatile isoprene emission are temperature-entrained and are likely photoperiod-gated.

Key words: circannual rhythm; MEP pathway; photoperiod; photosynthesis; qPCR; transcription; temperature compensation

Introduction:

Light-dependent foliar volatile isoprenoid biosynthesis and constitutive emission in plants occurs through the plastid-localised MEP (2-C-methyl-D-erythritol-4-phosphate) pathway. The intensity of volatile emissions is largely a function of light intensity, temperature, CO_2 and water availability (Monson & Fall, 1989; Sharkey *et al.*, 1991; Sharkey & Loreto, 1993). Like most plastidic biochemical pathways that are active during the sunlit hours of a day, isoprenoid emission in plants exhibits a diurnal rhythm and the process is circadian gated (Wilkinson *et al.*, 2006; Loivamäki *et al.*, 2007). Circadian regulation of isoprenoid emission impacts the formation of tropospheric ozone (Hewitt *et al.*, 2011). The phenomenon also has a circannual rhythm (summer maxima and winter minima) and understanding the biological mechanisms regulating the annual cycle has

implications for predicting emission consequences for regional weather and climate systems.

Volatile isoprenoid emission capacity of a species is determined by both aseasonal traits (growth habit and longevity) and seasonal traits such as leaf life span and photosynthetic capacity (Dani *et al.,* 2014a). Seasonal influences on the metabolic flux through the MEP pathway are well known (Owen & Peñuelas 2005; Rodríguez-Villalon *et al.,* 2009; Rasulov *et al.,* 2014). Most MEP pathway enzymes including isoprene synthase have long half-lives and substrate-level controls are implicated in dirunal regulation of emission (Wiberley *et al.,* 2009). Variation in substrate pool-sizes, feedback mechanisms, and post-translational modification of enzymes may play significant roles in pathway responses especially through developmental transitions (Smith *et al.,* 2004; Guevara-García *et al.,* 2005; Vickers *et al.,* 2011; Banerjee *et al.,* 2013; Ghirardo *et al.,* 2014; Wright *et al.,* 2014).

Expression of most genes of the MEP pathway is regulated by light, heat-shock, circadian and/or herbivore-elicitor transcription elements (Cordoba *et al.,* 2009; Meier *et al.,* 2011; Harrison *et al.,* 2013). Transcription, when it correlates with translation, could be a weak indicator of enzyme activity for the MEP pathway (Iijima *et al.,* 2004). Variation in the abundance of transcripts of *DXR* and *ISPS* (isoprene synthase) genes is linked with annual cycles in isoprenoid emission (Mayerhofer *et al.,* 2005). One of the key steps in the MEP pathway is its first step involving the formation of 1-deoxy-D-xylulose-5-phosphate (DOXP) by DOXP synthase encoded by the gene *DXS* (Wiberley *et al.,* 2009; Cordoba *et al.,* 2011; Han *et al.,* 2013). *DXR* (1-deoxy-D-xylulose 5-phosphate reductoisomerase) expression is responsible for accumulation of 2-C-methyl-D-erythritol-4-phosphate and is seen as an important step in isoprenoid biosynthesis (Carretero-Paulet *et al.,* 2006).

Given that the metabolic costs of transcription and translation have significant fitness costs (Stoebel *et al.,* 2008), regulation of the MEP pathway at the source (transcription) is more likely and cost efficient over seasonal time scales. Transcription, in general, is sensitive to both short-term dynamic environmental stimuli (over minutes) as well as incremental entrainment through seasons (Covington *et al.,* 2008; Harmer, 2009; Hoffman *et al.,* 2010; Igamberdiev *et al.,* 2014). Photoperiod is a predictable variable that enables plants to respond to seasons and thereby optimize their biosynthetic fluxes (Hay, 1990; Keskitalo *et al.,* 2005; Jackson, 2009; Velez-Ramirez *et al.,* 2011). For example, photoperiod influences developmental transitions from vegetative to reproductive phase in many annuals (Garner & Allard, 1920; Song *et al.,* 2013) and induction and breaking of bud dormancy in trees (Kramer, 1936; Bohleneus *et al.,* 2006). Light intensity, photoperiod and temperature interactions cause seasonal structural and chemical reorganization of photosynthesis in conifers (Öquist & Huner, 2003) and in many sub-tropical, temperate evergreen angiosperms (Hughes & Smith, 2007). Despite the involvement of many circadian (clock) elements in photoperiodic signalling pathway, photoperiod is likely to control plant responses independent of clock elements and temperature (Bradshaw & Holzapfel, 2010; Stoy *et al.,* 2014). In this context, phenological assumptions of global isoprenoid emission models and mechanisms underlying seasonal variability in isoprenoid emissions have not been satisfactorily explained especially in evergreen trees (Kuhn *et al.,* 2004; Harrison *et al.,* 2013; Unger *et al.,* 2013).

Species-specific temperature optima for growth and metabolism are an adaptive feature typical of geographically widespread and hyper diverse evergreens such as eucalypts (Scurfield, 1961; Paton, 1980). We expected that evergreen trees would exhibit significant

seasonal rhythms in their metabolism even though they do not undergo strict circannual leaf senescence. Down regulation of metabolic pathways during drier and/or colder seasons would be critical for plant survival and the mechanisms would be under stringent selection pressures and therefore are likely to be conserved across deciduous and evergreen habits (Adams *et al.*, 2004). We tracked isoprenoid emission, photosynthetic performance and real-time expression of selected key genes in evergreen eucalypts acclimated to distinct temperature and photoperiodic regimes. Based on reports that photoperiod has distinct effects on seasonal optimization of photosynthesis independent of temperature acclimation (Bamberg *et al.*, 1967; Bauerle *et al.*, 2012), we tested hypothesis that photoperiod could selectively regulate isoprenoid emission through controlling gene expression independent of temperature. The focus of the experiment was not to demonstrate circadian patterns in emission in eucalypts rather to separate the effects of photoperiod and temperature through seasons.

Materials and Methods:

***Plant material*:** Seeds of *Eucalyptus globulus* from the Australian Tree Seed Centre (Canberra) were germinated during winter (April) in Kraznozemic soil from Robertson area in New South Wales. 15-day seedlings were transplanted to pots (12 kg soil) containing the red soil mixed with Osmocote® slow release fertilizer. Plants were watered daily until the completion of the experiments. *E. globulus* was initially selected for the study (from a pool of 15 species) because it grew well in relatively small (12kg) pots and the seedling architecture was suitable for branch-level volatile sampling. However, it was observed that LD treatment (irrespective of temperature) induced faster transition from juvenile to adult phase in *E. globulus*, complicating the interpretation of volatile emissions and photosynthetic responses (see Appendix V). Transition from juvenile to adult leaves in

eucalypts is not a process of maturation. Both juvenile and adult leaves are fully expanded mature leaves and it is called *heterophylly*. We included *Eucalyptus camaldulensis* (which does not show heterophylly) as a control group during the winter experiment.

***Photoperiod and temperature treatment* (Table 1 and Figure 1):** After 60 d of growth under natural photoperiod at 25 ˚C/ 18 ˚C day/night temperature regime, plants bearing around ten fully developed leaves were segregated into four identical groups of four plants each. Each group was shifted to glasshouses with a natural or artificially extended photoperiodic regime (Fig. 1a, b; Table 1). Two treatment groups were at 25 ˚C/ 18 ˚C and the remaining two were at 33 ˚C/ 18 ˚C. Night temperature in all treatment groups was set at 18 ˚C (realistic during summer). Low night temperatures, typically <12 ˚C during winters in NSW, could have interfered with phytochrome signalling pathway complicating interpretation (Mølmann *et al.,* 2005). One group from each temperature treatment was subjected to long days for five months (simulated by low-fluence, cool-white fluorescent light, 40 µmol m^{-2} s^{-1}). The fluorescent light emission spectrum was rich in green (546nm; Hg vapour), red (610 to 660nm) and tiny bit of blue light. The LD (Long Day) glasshouses were oriented identically and the plants were randomised under the light bank. A continuously short-day acclimated treatment group was not included because we observed that eucalypts developed abnormal (non-pathogenic) intumescent blisters when grown under artificial lights in a growth cabinet (also see Pincard *et al.,* 2006). Instead, we employed naturally increasing (summer-ascent) daylengths as controls to the fluorescent light supplemented long-day (~16 h; Fig. 1b) treatment groups. Another set of seeds were germinated in mid-summer and shifted to a decreasing natural photoperiodic regime (winter-ascent) from February to July (Table 1).

Table 1: Details of day length set up at the experimental station (also see Figure 1B)			
Latitude and Longitude (33˚ 86′ S, 151˚ 21′ E)		Winter shortest day	Summer longest day
Sun rise		7 am	5:45 am
Sun set		5 pm	8 pm
Duration of sun light		~10 h	~14:15 h
Duration of fluorescent light	Natural photoperiod	Not applicable	
	Long day supplement	7 hours (3:30 pm to 10:30 pm)	
Duration of total photoperiod		~10 h	~16 h

Figure 1: Light regime

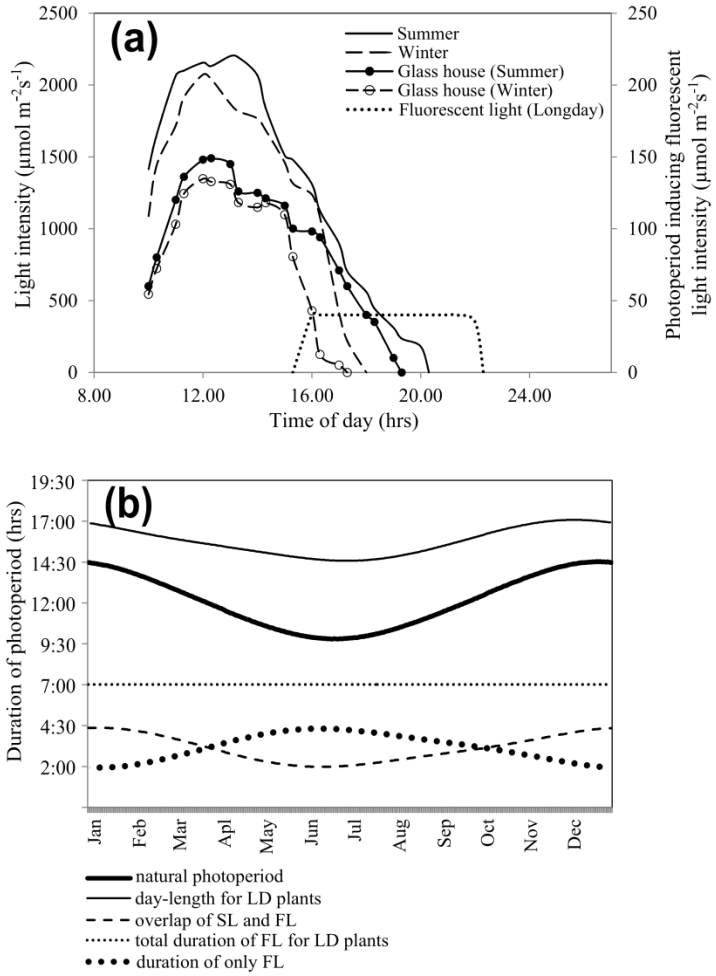

Figure 1: Light regime (A) Daily variation in solar light (SL) intensity in summer and winter at the experimental station. Long day (LD) treatment was achieved by low fluence fluorescent light (FL; 40 µmol m^{-2} s^{-1}) (B) Annual variation in duration of day length at the experimental location (Sydney, Australia). Duration of LD remained consistent (16:15 ± 0:30 hrs.) during summer-ascent (Jul to Jan) and winter-ascent (Feb-Jun). The constant 7 h line represents the fluorescent supplemental light given to LD plants during both summer and winter-ascent experiments. The dashed (-----) line represents the period of overlap between SL and FL that varied through the seasons since FL was turned on before sun set. The dotted (••••) line corresponds to the period when the LD plants received only FL every day.

***Photosynthesis and pigment concentrations*:** A LiCor 6400xt portable photosynthetic system (LI-COR, Lincoln, Nebraska, USA) was used to monitor the physiological status of the plant material. Measurements were carried out on three leaves per biological replicate between 11 am and 3 pm on bright sunny days during the months of December 2012 and January 2013 (Australian summer) and repeated in May to July 2013 (Australian winter). Cuvette CO_2 concentration was maintained at 400 µmol mol^{-1} and the light intensity was 1200 µmol m^{-2} s^{-1}. During summer the leaf temperature was set at 30 °C to reflect conditions of branch-level sampling. During winter, the leaf temperature was specific to the treatment group. Leaves were harvested immediately after sampling and total pigments were extracted in acetone. 0.5 g of leaf tissue was ground in a mortar using 5mL of 85% acetone with a pinch of acid washed sand and $MgCO_3$ (10 mg) and centrifuged at 8000 RPM. The extract was diluted suitably to determine chlorophyll and carotenoid concentrations by spectrophotometry (Thermo Scientific, USA) following Lichthenthaler and Wellburn (1983).

***Volatile isoprenoid sampling*:** 25 L capacity Tedlar® bags (Sigma) were modified to make branch-level volatile collection chambers with a PTFE (polytetrafluoroethylene) base for air tight sealing. An empty chamber was flushed with clean-air at a high flow rate (20 L min^{-1}) for 5 min. A branch was inserted into the chamber and sealed around at the base. Plants were provided with natural sun light from 10 am until 5 pm during sampling and branch temperature was 30 ± 2 °C in summer. In winter, a LI6400 XT portable gas exchange system was suitably modified to sample volatiles (using volatile free humidified air mixed with high purity CO_2) directly from the leaf cuvette onto the thermal adsorbent bed as described in Dani *et al* (2014b). Volatile emissions were sampled from two to three

leaves (winter) or two to three branches (summer) per biological replicate (N = 4 to 5) per treatment group. During cuvette sampling (winter) leaf temperature was maintained in accordance with the temperature acclimation of the treatment group and light intensity was maintained at 1200 µmol m^{-2} s^{-1} irrespective of the time of day.

***RNA extraction and real time qPCR*:** The leaves were collected just after volatile sampling (winter) between 11 am and 3 pm, tissues were flash frozen in liquid nitrogen and stored at -80 ˚C until further use. To extract RNA from eucalyptus leaves comprising a large amount of polyphenolic substances (inhibitors of cDNA synthesis and qPCR), a conventional RNA extraction protocol was preferred to popular commercial extraction columns/kits. High quality RNA was extracted in an extraction buffer comprising cetyl trimethyl ammonium bromide (CTAB), polyvinyl pyrolidone K30 (PVP) (modified from Chang *et al.,* 1993; Zeng & Yang 2002). RNA was treated with DNase I (Sigma) and RNA quality was checked through denaturing agarose electrophoresis and A_{260nm}/A_{280nm} ratio was always >1.8 (often >2.0; tested using an Eppendorf biophotometer). First strand cDNA synthesis was performed at 50 ˚C using the Transcriptor cDNA synthesis kit (Roche, GmbH) using anchored oligo dT_{18} primer on 1µg total RNA as the starting template and 10U of Transcriptor reverse transcriptase. Real time qPCR was performed using a Light Cycler 2.0 (Roche®). Reactions were setup in triplicates for each primer pair per tissue sample (RNA pooled from three leaves within plant) using the SYBR Green Fast Start Master Kit (Roche®) starting with 5 ng of cDNA template and transcript specific primers (Table S1, Appendix V). The amplification cycle had the following programme 95 ˚C (10 min); 40 cycles of [95 ˚C (20 s) + 54 ˚C (30 s) + 72 ˚C (20 s)] at 20 ˚C/s, 72 ˚C (20 s) at 0.1 ˚C/s, 35 ˚C (30 s). Recently sequenced draft genome of *Eucalyptus grandis* (Eucagen project; Myburg *et al.,* 2014) and the available cDNA and genomic sequences at the

NCBI's Genbank from various eudicots (especially *Populus* and *Quercus*) were employed to design qPCR primers (Table S1, Appendix V). Wherever possible primers spanned an exon-exon junction and most amplicons were closer to the 3′ end of their respective cDNAs to overcome 3′ bias of oligo dT cDNA pool. α-tubulin, ubiquitin, actin and 18S rRNA were tested as candidate reference genes. Actin and 18s rRNA were selected as internal reference genes. We selectively monitored *DXS* and *HDR* transcript levels (representing the MEP pathway) since DXS enzyme catalyses the rate limiting step of the MEP pathway (Estévez *et al.*, 2001) and HD reductase catalyses the formation of DMAPP, the substrate for isoprene synthase. In addition, light induced transcript abundance of *DXS*, *HDR* and *ISPS* exhibit a distinct single peak per day (around midday; Wiberley *et al.*, 2009) and the abundance of *DXS and HDR* strongly correlates with their enzyme levels (León & Cordoba, 2013). Standard quantification curves for two reference genes and eight target genes were generated over a cDNA template working concentration range of 5pg to 50ng and qPCR efficiency (Table S1, Appendix V) was calculated using the Light Cycler Software v4.0.

***GC-MS analysis*:** A Shimadzu GCMS-QP 2010 machine was modified to accommodate a customised thermal desorption and cryo-focussing unit feeding into the injection port. Ultrapure helium (BOC) was used as the carrier gas. Isoprene (analytical grade, Sigma) standard samples injected into sterile Tedlar® bags, and adsorbed onto TD tubes were used to calibrate the instrument at regular intervals. Volatiles were desorbed from the TD tube at 220 $^{\circ}$C at a flow rate of 10 mL min^{-1} for 5 min and simultaneously cryo-focussed by passing the desorbed volatiles through a stainless steel loop immersed in liquid nitrogen. The sample was directed through a programmed temperature vaporisation (PTV) injection port by quickly heating the cryo-focussing loop to 260 $^{\circ}$C using a heat gun. A 30 m, 25 mm ID, 25 µm RTX-5 Sil MS (Restek) capillary column was used for GC. The

temperature regime for GC run was 28 $^{\circ}$C (3 min) to 110 $^{\circ}$C (3 min) at 5 $^{\circ}$C min^{-1} and finally to 180 $^{\circ}$C at 5 $^{\circ}$C min^{-1}. Appropriate reference standards were run and volatiles were quantified following Dani *et al* (2014b).

***Statistical analyses*:** Isoprene emission was sampled from two to three leaves (winter) or two to three branches per biological replicate (N=4 to 5) per treatment group. Significance of the phase of the circadian peak (Fig. 2(a)) was computed using *t* tests for the time interval (within the temperature group) since circadian rhythmicity was established in other similar systems and not assumed (Refinetti *et al.*, 2007). Data from several days were stacked to obtain a single synchronised cycle. The stacking of data was done for hourly time intervals between 12 noon and 3 pm except at the beginning *i.e.*, from 10 am to 12 noon and then at the end of the day from 3 pm to 5 pm (Fig. 2(b)) since isoprene sampling duration per sample was doubled for morning and evening samples. Data were normalised within each treatment group and cosinor analysis (Cornelissen, 2014) was performed using a Matlab® programme (Heart, 2008; Mathworks, MA, USA; Fig. 2(c)). Actin (medium copy) and 18s rRNA (high copy) were selected as candidate reference standards although only 18s rRNA transcript abundance was used to quantify relative fold change in transcripts by comparative C_T method ($2^{-\Delta\Delta CT}$, Schmittgen & Livak, 2008).

Results:

***Growth and photosynthesis*:** Cool temperatures coupled with long-days are expected to increase biomass accumulation and any significant increase in temperature is expected to mask the positive effect of long-days on biomass accumulation (Hunter *et al.*, 1974).

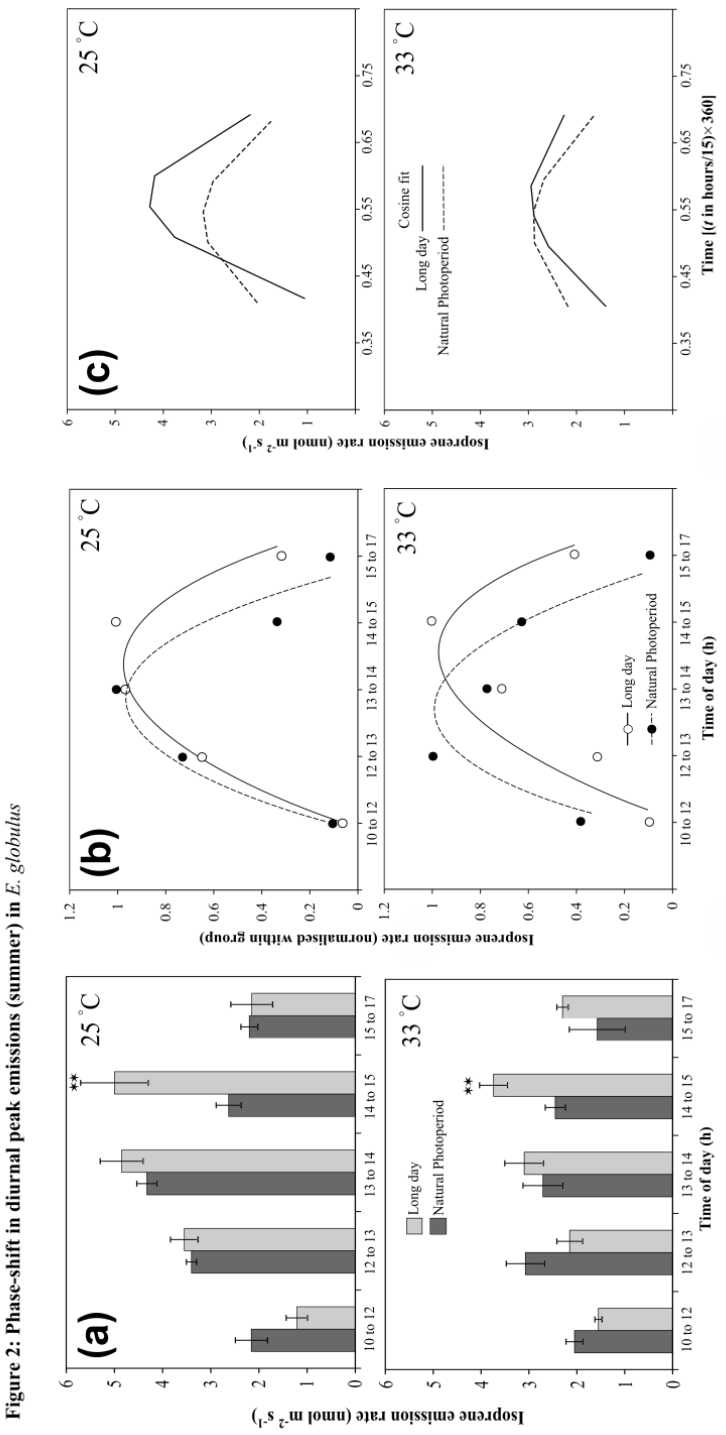

Figure 2: Long-day and temperature effects on the diurnal phase of isoprene emission in *E. globulus* during summer-ascent: Top panel at 25 ˚C and the bottom panel at 33 ˚C (a) Original emission data (N=4; mean ± 1 SE; $^{**}P$<0.05). Emission rates at 25 ˚C are higher in absolute terms than those at 33 ˚C because the branch temperature was 30 ± 2˚C for both treatment groups during sampling and as a result, seedlings from 25 ˚C were exposed to higher temperatures during sampling. (b) Data are normalised within each treatment group. Phase shift is captured by fitting second degree polynomial curves (least-squares regression, r^2=0.76 to 0.96). (c) Single component cosine fits for isoprene emission rates (differences significant at α=0.05).

Table 2: Photosynthetic performance of *Eucalyptus globulus* under two different photoperiodic regimes in winter and in summer

Growing temperature		25 ˚C				33 ˚C			
Season		Winter		Summer		Winter		Summer	
Photoperiod group		Natural photoperiod	Long day	Natural photoperiod	Long day	Natural photoperiod	Long day	Natural photoperiod	Long day
Net assimilation rate (μmol $m^{-2}s^{-1}$)		11.2 ± 1.06	6.9 ± 0.90*	10.1 ± 0.83	9.3 ± 0.74	10.4 ± 1.25	10.6 ± 1.43	12.4 ± 0.67	10.2 ± 0.83
Stomatal conductance (mol $m^{-2}s^{-1}$)		0.210 ± 0.043	0.116 ± 0.043*	0.184 ± 0.009	0.192 ± 0.012	0.180 ± 0.034	0.208 ± 0.058	0.205 ± 0.011	0.188 ± 0.009
C_i (at C_a=400 μmol mol^{-1})		261 ± 22	264 ± 17	217 ± 8	226 ± 35	269 ± 17	265 ± 26	236 ± 13	217 ± 8
Transpiration rate (mmol $m^{-2}s^{-1}$)		4.2 ± 0.55	2.9 ± 0.53*	2.8 ± 0.25	2.6 ± 0.08	5.1 ± 0.71	5.1 ± 0.82	2.6 ± 0.09	2.8 ± 0.25
Leaf temperature (˚C)		22.7 ± 0.8	23.8 ± 0.9	ND		28.5 ± 0.6	29.2 ± 0.4	ND	
Relative humidity (%)		38 ± 3	38 ± 2	35 ± 1	35 ± 1	38 ± 2	37 ± 3	36 ± 1	34 ± 3
Chlorophyll concentration (μg g^{-1} FW)	Chl *a*	722 ± 105	472 ± 61*	ND		663 ± 95	643 ± 38	ND	
	Chl *b*	240 ± 29	155 ± 20			255 ± 60	264 ± 37		
Total carotenoids (μg g^{-1} FW)		147 ± 11	103 ± 7*			125 ± 46	113 ± 22		

*P<0.05; ND: Not determined; †Branch-level measurements

This prediction was confirmed in *E. camaldulensis* during winter-ascent but not in *E.globulus.* LD treatment had a significant positive effect on total vegetative growth (mean heights) in *E. globulus* at both 25 ˚C and 33 ˚C only during summer-ascent (Fig. S1,

Appendix V). Neither LD nor warmer temperature acclimation affected net assimilation rates during summer-ascent (Table 2).

Artificially imposed LD during winter-ascent caused significant decline in net assimilation rate in *E. globulus* at 25 ˚C but the seedlings acclimated to 33 ˚C and LD did not show any change. There was no significant change in net assimilation rate across treatment groups in *E. camaldulensis* (with adult foliage) during winter-ascent (Table S2, Appendix V). LD plants had less chlorophyll per unit area (not determined directly but the leaf area was relatively bigger) but with no total change in chlorophyll (determined only during winter; Table 2). But the decrease in chlorophyll was statistically significant only in *E. globulus* at 25 ˚C under LD and we believe it was an anomalous response (see discussion). Total carotenoid content (a product of an extended MEP pathway) was insensitive to both temperature and photoperiodic acclimation in *E. globulus* (Table 2).

***Volatile emissions*:** *Eucalyptus globulus* seedlings acclimated to natural photoperiod at both 25 and 33 ˚C showed a significant diurnal peak between noon and 2 pm during summer-ascent. During the same period, seedlings experiencing continuous LD (~16 h) showed a significant temporal delay in their peak daily isoprene emission rate occurring between 1 and 3 pm (Fig. 2). The peak-broadening/-shifting effect persisted irrespective of temperature acclimation. During the summer-ascent experiments, there was unavoidable temperature variation at the branch-level due to variation in radiant sunlight. Therefore, the measurements made in summer could not test whether LD also influenced the absolute emission rates. During winter, leaf-level emission measured at constant light and treatment-specific temperature showed that interaction between LD and warmer temperature (25 → 33 ˚C) in *E. globulus* caused an absolute increase in isoprene emission rate (Fig. 3a). By contrast, LD at 25 ˚C caused a significant increase in isoprene emission

rate in *E. camaldulensis* (Fig. 3a) and the effect of LD was not significant at 33 ˚C. Constitutive monoterpene emission decreased in absolute terms in plants under LD although the difference was statistically significant only for *E. globulus* acclimated to 33 ˚C (Fig. 3b).

Figure 3: Isoprenoid emission rate during winter-ascent: Interactive effects of photoperiod and temperature acclimation

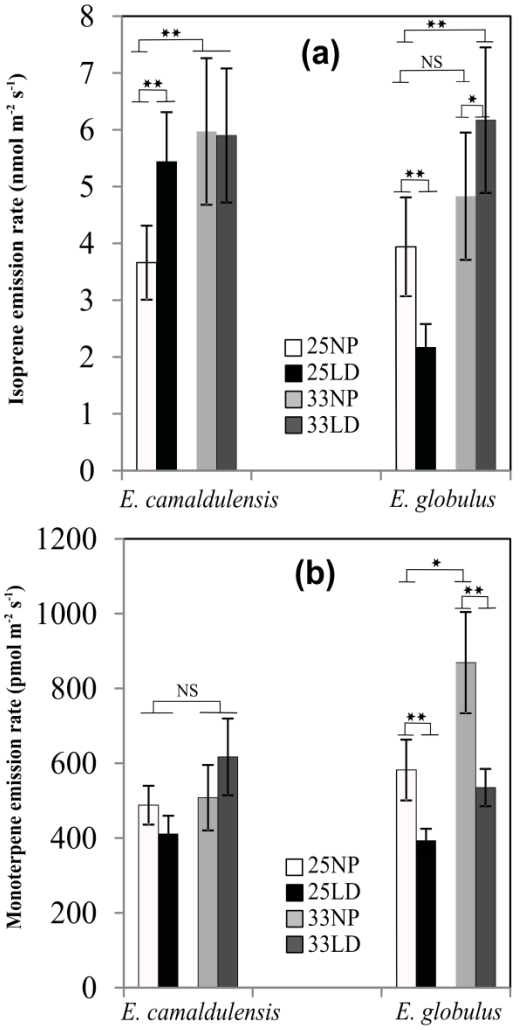

Figure 3: Interactive effects of photoperiod and temperature acclimation on (A) Isoprene emission rate and (B) Constitutive monoterpene emission rate in *E. globulus* (juvenile foliage) and *E. camaldulensis* (adult foliage) during winter-ascent. Each bar corresponds to mean ± 1 SE (*N*=4 to 5; also see Table 2).*P<0.1, **P<0.05; NS is not significant; *t*-test at α=0.05.

Figure 4: Transcript abundance during winter-ascent

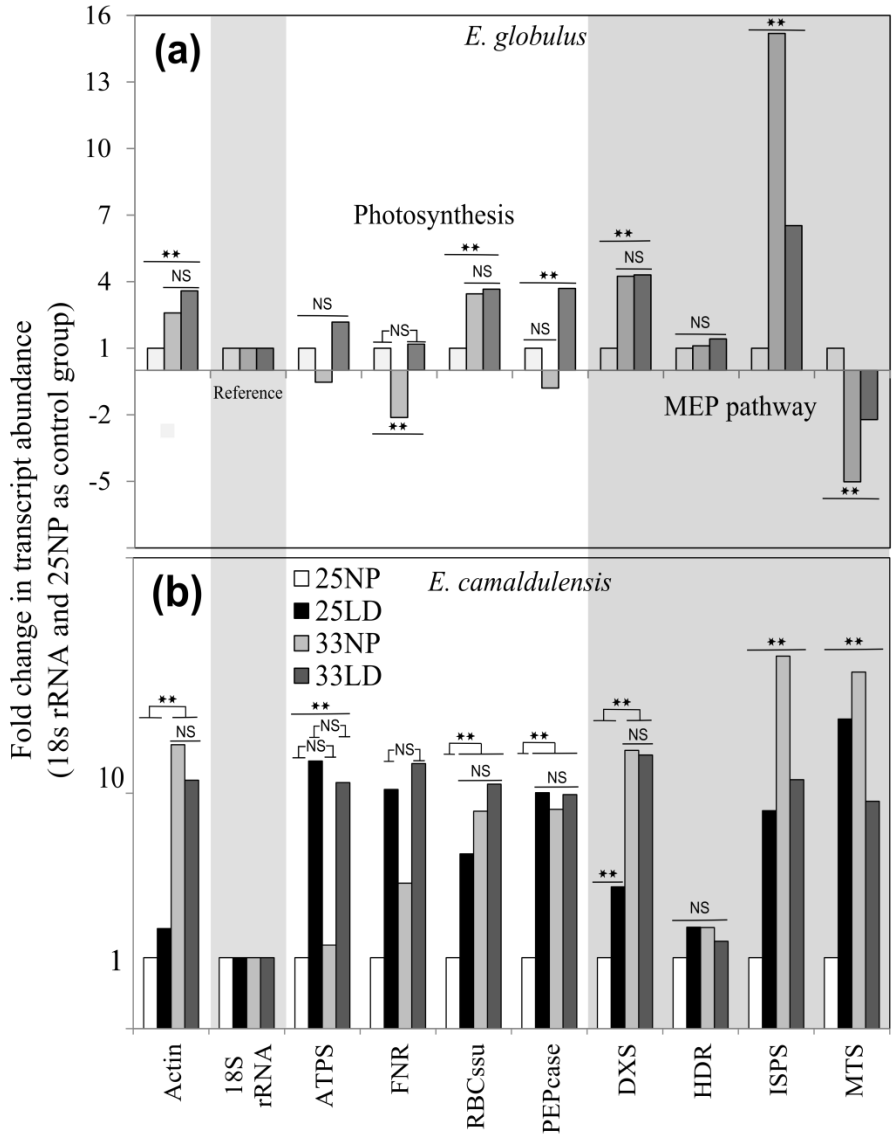

Figure 4: Fold-change in the transcript of selected genes from photosynthesis and the MEP pathway during winter-ascent in (A) *E. globulus* and (B) *E. camaldulensis*. Fold-change is calculated by keeping the estimates from control group (25 ˚C, natural photoperiod) and 18S rRNA abundance as reference. Each column represents fold-change values from triplicate qPCRs done using RNA extracted from nine leaves per treatment group (*N*=4 to 5). The symbol ** indicates significant change and NS is not significant. Note: Y-axis in (A) is linear and it is logarithmic in (B). The fold-changes for *E. globulus* (25 ˚C; LD) is not presented in Fig 4 (a) since it had significantly different responses. The data is provided in Appendix V and explained under discussion section.

***Transcript abundance (during winter-ascent)*:** Compared to control (natural photoperiod, 25 ˚C), in *E. globulus*, transcripts of nuclear encoded small subunit of Rubisco significantly increased under warmer acclimation while those of ferredoxin-NAD reductase (*FNR*) decreased and that of γ-subunit of chloroplastic ATP synthase (*ATPS*) remained unchanged (Fig. 4). While warmer temperature on its own induced increased transcription of phospho-enol pyruvate carboxylase (*PEPC*) and *DXS* in both the species, abundance in these transcripts also increased due to LD (at 25 ˚C) alone in *E. camaldulensis* but not in *E. globulus* (Fig. 4). In *E. globulus* consistently across treatment groups any increase in *ISPS* transcripts and isoprene emission rate was associated with a marked decrease in *MTS* transcription (Fig. 4a) and constitutive monoterpene emission rates (Fig. 3b). *HDR* (4-hydroxy-3-methylbut-2-enyl diphosphate reductase) transcripts did not change in both species under all treatments. Both *ISPS* and *MTS* transcripts increased in response to warmer temperature acclimation in *E. camaldulensis* (without LD) but only isoprene emission rate increased and not that of monoterpenes (Fig. 3a). Transcripts of *FNR* and *ATPS* significantly increased in *E. camaldulensis* only at 33 ˚C when combined with LD (Fig. 4b).

Discussion:

It is known that differences in the sensitivity of transcriptional regulatory elements to key environmental cues plays a significant role in modulating circadian gated plant metabolic pathways (Michael *et al.*, 2003; Farré & Weise, 2012). We hypothesised that photoperiod influences seasonal regulation of foliar volatile isoprenoid emission independent of temperature through transcriptional regulation of core-photosynthesis genes and selected MEP pathway genes.

We found that irrespective of steady-state temperature acclimation, *Eucalyptus globulus* seedlings grown under artificially induced long day (LD) exhibited altered (delayed) phase in their diurnal emission rhythm (Fig. 2). LD is shown to cause phase-shifts in transcription of a gene coding for a chlorophyll binding protein in *Arabidopsis* (Millar and Kay, 1996). Similarly, transgenic isoprene emitting tobacco (under constitutive 35S promoter) was seen to sustain diurnal rhythm in their emission only under long-day treatment (Vickers *et al.*, 2011). Our observation of altered relative peaking time in emission in a eucalypt under LD is consistent with our understanding that the phase of circadian rhythms is a function of photoperiod and light transduction (Pittendrigh & Minis, 1964; Heide, 1977; Somers, 1999).

Our findings also need to be explained in terms of function of isoprene emission in emitting plant genera and we propose that the sensitivity of the diurnal phase in emission to LD, and thus to summer, suggests a longer-term (evolved over many millions of years) link between isoprene emission rates and ground-level ozone concentrations. It is known that isoprene emission mitigates ozone stress in emitting plants (Loreto and Velikova, 2001; Behnke *et al*. 2009; Fares et al. 2009). It is also known that daily mean ground-level ozone concentration (excluding anthropogenic input) is not only higher during summer relative to winter, but also follows a broader and delayed diurnal peaking in summer due to higher solar irradiation and warmer temperatures than in winter (Khoder, 2009; Sharma *et al.*, 2012).

While we acknowledge that we used branch-level measurements and it had limited capability to maintain constant leaf temperature during sampling, the consistency of the

phase-shift in plants acclimated to both 25 and 33 ˚C (prior to sampling) indicated temperature compensation, which is a feature of strictly circadian gated metabolic processes (reviewed in Webb, 2003). The result also suggested the existence of an independent, master photoperiodic clock that regulate the MEP pathway, most likely through *trans*-acting elements that are triggered by integration of the total duration of light perceived by the plants per day. Increased volatile emission rates occurring at two different temperatures for the two species pointed to a broad entrainment of the MEP pathway by latitude-specific temperature and photoperiodic signal processing. Temperature (heat) on its own may override LD controls by causing bursts in emission especially during sun-flecks or short-term heat stress over short-term depending on the available DMAPP substrate pool sizes.

Seasonal variation in light intensity and temperature cause modulation in photosynthetic electron transport (Martin *et al.*, 1978) and enzyme activities (Gazelius & Hallen, 1980). Although a correlation is known between Rubisco activity and content with its mRNA abundance (Jiang *et al.*, 1993), in our experiments the difference in photosynthetic variables was not significant across treatment groups suggesting seasonal thermal entrainment of chloroplastic processes, except in *E. globulus* during winter, 25 ˚C, LD; Table 2, Table S2 in Appendix V). We attributed the significant decline in net assimilation rate in *E. globulus* (winter, 25 ˚C, LD) to chlorosis (Table 2), which is common in plants including many eucalypts sensitive to artificial light (Withrow & Withrow, 1949; Scurfield, 1961). Interestingly, the transcripts of the representative thylakoid membrane proteins responded positively to LD acclimation, suggesting transcriptional regulation of photosynthetic light reactions by photoperiodic signalling. Preservation of photosynthesis

and chlorophyll content in *E. globulus* acclimated to 33 ˚C suggested seasonal thermal entrainment of chloroplastic processes.

Physical factors such as light and drought have a complex interactive influence on photosynthesis and emissions (Misson *et al.*, 2006; Staudt & Lhoutellier, 2011; Dani *et al.*, 2014b). However, a more recent model identified canopy temperature as the main determinant of seasonal variation in community-level isoprene emission rates (Foster *et al.*, 2014). We found that *DXS* and *ISPS* were synchronously up-regulated in leaves emitting isoprene at significantly higher rates (Fig. 3, 4). Temperature had the most significant positive effect on both *ISPS* transcription (>10 fold increase) and isoprene emission rate in eucalypts (Fig. 3, 4). Hierarchical and modular organization is a feature of transcription networks controlled by light and temperature (Franklin, 2008; Erwin & Davidson, 2009). Co-ordinated transcriptional up regulation of *DXS* and *ISPS* genes by photoperiod and temperature reflects a 'selectively' modular organization of the MEP pathway transcription, where groups of genes are co-regulated irrespective of their respective position in the pathway.

Circadian regulatory elements are highly conserved across plant groups, yet photoperiodic regulatory circuits have significantly diverged (Hayama *et al.*, 2003; Song *et al.*, 2010; Campoli *et al.*, 2013) and have acquired novel functions through the evolutionary history of land-plants (Kubota *et al.*, 2014). Unlike deciduous trees from the high latitudes, evergreen eucalypts seemed to have evolved greater sensitivity to annual temperature cycles than to photoperiod (Paton, 1978). The fact that in our experiments emission rates increased under LD (with marginally less chlorophyll per leaf area) suggested that increased emission was a true response to LD, given that positive

correlations exist between nitrogen and chlorophyll content and further between chlorophyll content and emission rates (Possell *et al*., 2004; 2005). In another study, isoprene emitting transgenic tobacco acclimated to LD, emitted isoprene at twice the rate of those under short-day treatment (Vickers *et al.*, 2011). Interestingly, transcripts of *ISPS* and *MTS* declined in 33LD in both species while *DXS* did not. Under normal photoperiod, warm temperature acclimation could lead to a bigger increase in transcription (as a general rule) and LD signals are needed to optimise the pathway over seasonal time-scales. Therefore, we propose that the amplitude of emission (seasonal maxima and minima) is entrained by temperature and is photoperiod-gated. Quantifying species-specific temperature optima for photosynthesis and more obviously the MEP pathway could provide a mechanistic basis to global seasonal emission oscillations.

Conclusion:

Light-dependent plant metabolic pathways are circadian gated and regulated by rhythmic expression of multiple genes. We aimed to quantify the relative effects of photoperiod and temperature on seasonal variation in plant volatile isoprenoid emissions. The circadian rhythm of volatile emission is temperature compensated. Acclimation to long days (induced through low fluence light) coupled with warmer temperature causes increased emission rates without causing any significant change in photosynthetic performance. Temperature induced transcription of *DXS* and isoprenoid synthases is one of the mechanisms of regulating circannual (not circadian) variation in plant isoprenoid emission. We conclude that temperature is the principal *time-giver* that entrains circannual emission rates and it is likely gated by a photoperiodic clock. This also means that increasing global mean temperature could interfere with the photoperiod (unchanged) signalling in major emitting tree genera altering their seasonal emission responses.

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Chapter 6

Evolution of isoprene emission capacity in plants[6]

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[6] **Contributions**: The hypotheses proposed in this article was conceived and developed by KGSD. He first proposed the idea during the MQU international isoprenoid workshop held in Macquarie University in November 2011. KGSD collated the data set, analyzed it and wrote the opinion. KGSD had important discussions with ICP, BJA, Roger Hiller and Francesco Loreto (Italy). BJA corresponded with the editor and helped refine/revise the flowchart presented in this article. All authors had editorial inputs.

Opinion

CellPress

Evolution of isoprene emission capacity in plants

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Light-dependent *de novo* volatile isoprene emission by terrestrial plants (approximately 2% of carbon fixed during photosynthesis) contributes as much as 0.5 Pg C/year to the global carbon cycle. Although most plant taxa exhibit either constitutive or inducible monoterpene emissions, the evolution of isoprene emission capacity in multiple lineages has remained unexplained. Based on the predominant occurrence of isoprene emission capacity in long-lived, fast-growing woody plants; the relationship between 'metabolic scope' of tree genera and their species richness; and the proposed role of high growth rates and long generation times in accelerating molecular evolution, we hypothesise that long-lived plant genera with inherently high speciation rates have repeatedly acquired and lost the capacity to emit isoprene in their evolutionary history.

Plant isoprene emission: *mysterium tremendum et fascinans*

Biogenic volatile organic compounds (BVOCs), such as isoprene (C_5H_8) and monoterpenes ($C_{10}H_{16}$), together referred to as isoprenoids, and their oxidation products in the atmosphere, have a climatically significant influence on secondary organic aerosol formation (e.g., [1]), tropospheric ozone production (e.g., [2]), and the atmospheric lifetime of methane (e.g., [3]). As the atmospheric burden of greenhouse gases increases, and global mean temperatures rise, it is predicted that plant isoprenoid emission levels could provide a significant contribution to the positive feedback between biogeochemical processes and climate change (e.g., [4]).

Volatile isoprenoid emissions from plants have been studied since the 1960s (e.g., [5]), but even during the 1990s, the phenomenon of plant isoprenoid emission was seen as an enigma [6]. Nearly two decades after discovering a new isoprenoid biosynthetic pathway (1-deoxy-D-xylulose-5-phosphate/methyl erythritol phosphate; DOXP/MEP) in plant chloroplasts, scientists still wonder why isoprene emission by *de novo* synthesis has evolved only in some plant taxa [7]. Isoprenoid biosynthesis in plants can occur through two spatially separated pathways *viz.* the cyanobacterial DOXP/MEP pathway in plastids and the archaeal mevalonic acid (MVA) pathway in the cytoplasm [8]. It is still not clear what regulates the metabolic cross-talk between the two pathways. Experiments designed to characterise the MEP pathway have not yielded consistent responses to similar treatments across plant taxa [9], thereby offering few clues to the evolutionary events that gave rise to the phenomenon of isoprene emission.

Metabolic regulation of isoprene emission and utilitarian arguments

The processes of synthesis and emission of isoprene are biochemically expensive and energy dependent [10] and thus, *ceteris paribus*, expected to be under negative selection pressure. Studies on the impact of CO_2 enhancement on plant VOC profiles show that high CO_2 concentrations

Glossary

ceteris paribus: Latin for 'other things being equal'.
Isoprenoids: 5–15 carbon volatile hydrocarbons *viz.* isoprene (C_5H_8), monoterpenes ($C_{10}H_{16}$),and sesquiterpenes ($C_{15}H_{24}$); a subclass under a large group of organic molecules called terpenoids.
J_{max}: maximum electron transport rate by photosystem II.
Metabolic scope: a large difference between resting and maximum metabolic rate of a genus increases the number of metabolic niches and results in species richness [27].
mysterium tremendum et fascinans: Latin for 'a mystery before which man both trembles and is fascinated'.
Neofunctionalisation: acquisition of novel function by a newly acquired duplicate of an existing gene (thus losing original function) through preservation of favourable mutations.
Orthologue: genes in different species that evolved from a common ancestral gene by speciation. Orthologues tend to acquire the same function during the course of evolution.
Paralogue: genes related to each other through duplication. Paralogues can evolve new functions.
Phanerozoic: the geologic period that began approximately 570 million years ago (mya). It encompasses three geologic eras: the Palaeozoic (570–250 mya), Mesozoic (250–65 mya), and Cenozoic (65–0 mya).
Poikilohydry: ability to maintain water status in accordance with changes in the surrounding environment.
Trait vis-à-vis process: a trait is a genetic and/or physical, and/or physiological manifestation of a biological process; for example, speciation (a facet of evolution) is a process applicable to all genera, whereas speciation rate is a trait characteristic of a genus.
Terpenoids: a large group of organic compounds (>20 000 different molecules) that come under the class of secondary metabolites. The term usually refers to higher order hydrocarbons ($>C_{20}H_{40}$); for example, carotenoids, phytol chain of chlorophyll, brassinosteroids, and natural rubber.
V_{cmax}: maximum carboxylation rate by Rubisco.

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(at least in the short term) inhibit isoprene emission. Consequently, it has been proposed that isoprene biosynthesis consumes reducing power left unused by photosynthesis at ambient or subambient CO_2 levels, because an increased carbon reduction rate under CO_2 fertilisation consumes more NADPH and ATP (e.g., [11]). Isoprenoid biosynthesis has also been viewed as a metabolic 'safety valve' [12] effected by the flux of phosphoenolpyruvate between the chloroplast and the cytoplasm. Some see isoprenoid emission as an opportunistic utilisation [13] of the MEP pathway metabolites not used for hormone and pigment biosynthesis. Considerable evidence suggests that isoprene emission: (i) protects plant cells against transient high temperatures (the thermotolerance hypothesis) [14,15]; (ii) facilitates thylakoid membrane stability at high temperatures [16]; (iii) protects the photosynthetic apparatus through quenching ozone-induced free radicals [17]; and (iv) has a role in drought tolerance [18]. Here, we propose a framework for hypotheses on the evolution of isoprene emission in plants, postulating that fast-growing, long-lived, highly diverse woody plants are more likely to be isoprene emitters.

Somatic mutations and species richness in perennial plants

Arborescence is thought to have evolved many times among land plants. Given the lack of clarity as to whether woodiness is more ancient than herbaceousness in angiosperms [19,20], it is noteworthy that isoprene emission capacity is predominantly observed in long-lived woody plants [21] and a few perennial grasses, ferns, some aquatic plants, and mosses [22], which curiously are also long lived. It has been suggested that fast-growing trees in plantations are significant contributors to global isoprenoid emissions [23] because of their sheer numbers and vast cross-continental geographic distribution.

One of the consequences of being perennial is that trees are exposed to seasons and, therefore, more extreme weather events throughout their life cycles compared with herbaceous relatives [24]. Somatic mutations in plants are important because the reproductive organs (e.g., flowers in angiosperms) come about through morphogenesis of somatic meristems. Despite slow cell division and growth rate among large trees, their long life span results in larger number of somatic (premeiotic) cell divisions per generation relative to herbaceous species (e.g., [25,26]). When such long-lived plant genera also have high growth rates, they are likely to accumulate more somatic mutations per generation more quickly than an average genus. Generally, evolution in protein function is seen as a function of the rate and preservation of mutations in germ lines and is linked to species-specific metabolic rates and generation time [27–30]. A genus that has a large metabolic scope [27] will provide opportunities for many species of that genus to develop distinct ecophysiologies and, thus, occupy diverse habitats. This implies that high metabolic and growth rates have made some genera speciose. When such speciose genera are trees (long lived), they are predisposed to accumulate mutations that lead to the acquisition (or loss) of rapidly evolving traits, such as isoprene emission capacity (Box 1). The role (if any) of isoprene emission in diversification of emitting genera is difficult to test [31] because species richness within plant genera is primarily driven by (i) pollination mode (biotic versus abiotic); and (ii) growth habit (woody versus herbaceous; relevant to our hypothesis) [32].

Most gymnosperms are woody perennials. Despite slow growth rates and low diversification rates [33,34], gymnosperms (and almost all plants) have evolved a capacity to emit foliar monoterpenes both stored and synthesised *de novo*. We propose that the fundamental causes are: (i) relative to angiosperms, gymnosperms have lower V_{cmax} and higher J_{max} [35], which indicates a favourable needle energy status to sustain isoprene and constitutive monoterpene emission. Gymnosperms also have a higher tendency than angiosperms to leak electrons towards oxygen-dependent reactions in the chloroplast (especially to the Mehler reaction) to avoid over-reduction of the electron transport chain [36], indirectly suggesting that gymnosperms have excess electrons relative to the demand for carboxylation; and (ii) highly specialised functional roles for foliar storage and emission of monoterpenes in antiherbivory and allelopathy [37] could have increased the 'phylogenetic inertia' of the monoterpene emission trait, because the overall fitness of such traits is linked to other complex coevolved behaviours (e.g., [38]). Isoprene serves many physiological functions, some of which are experimentally proven. However, its role in complex ecological interactions remains ambiguous [37].

Gene and genome duplications: molecular evolution and functional diversification

The capacity to synthesise and emit isoprene is due to the presence of an isoprene synthase (*ISPS*) gene and the translated enzyme in the MEP pathway [39,40]. The *ISPS* gene is present only in some plant genera. Many plant genes are part of their own huge gene families and terpenoid synthases (comprising isoprene and monoterpene synthases) are no different [41]. It has been suggested that monoterpene synthases in angiosperms and gymnosperms are orthologues and isoprene synthase is a paralogue of ancestral monoterpene synthase [42,43] (Figure 1). The circumstances surrounding the origin of *ISPS* could explain why it repeatedly appears and disappears from genomes.

High sexual reproductive frequency and genetic recombination may mean higher rates of new gene acquisition events among annuals [44]. However, recruitment of new genes (which could be duplicate genes) through sexual reproduction does not necessarily translate into neofunctionalisation of duplicate genes, because it needs preservation of mutations. Our hypothesis emphasises the importance of premeiotic cell divisions and somatic mutations in increasing the probability of such events due to long generation times in perennial plants [26]. Most known gene duplications in plants have come about through whole-genome duplications and a significant number (15–20%) of them involve dispersal of tandem and segmental repeats [45]. Duplications in some loci are more tolerated in plants depending on the fitness and selection pressures on the metabolic pathway network to which they belong [46]. Enzymes involved in the MEP pathway are not

Box 1. Species richness and occurrence of isoprene emission in various plant lineages

Molecular marker-based phylogenies for selected angiosperm families, pteridophytes, and gymnosperms were adapted from literature (for references, see Table S4 in the supplementary material online). The phylogenetic trees (Figure I) were simplified to represent contrasting sister groups (at the level of genera, both woody wherever possible) such that one clade emitted isoprene (in red) and the other did not. Isoprene-emitting capacities were identified and verified using the Lancaster University emission database (http://www.es.lancs.ac.uk/cnhgroup/download.html) and published literature. Species richness of selected tree genera was obtained from *The Plant List* of the Missouri Botanical Gardens and the Royal Botanic Gardens, Kew (http://www.theplantlist.org/). A Wilcoxon's two-tailed signed-ranks test was performed with cumulative number of species for emitting and nonemitting sister clades treated as paired observations ($n = 14$; Table S4 in the supplementary material online).

The hypothesis that isoprene emission occurs in genera that are significantly more speciose than their nearest sister genera is accepted ($P<0.01$). It is important to remember that species richness is just an indicator that finds a pattern in occurrence of isoprene emission where no pattern is apparent. Speciation does not lead to acquisition of isoprene emission but both features seem to coevolve in long-lived genera. Although adaptive selection has a role in sustaining isoprene emission through changing climate regimes, the capacity to emit isoprene appears to have arisen repeatedly and independently due to biological factors, such as plant growth rate, growth habit, longevity, and metabolic pathway plasticity.

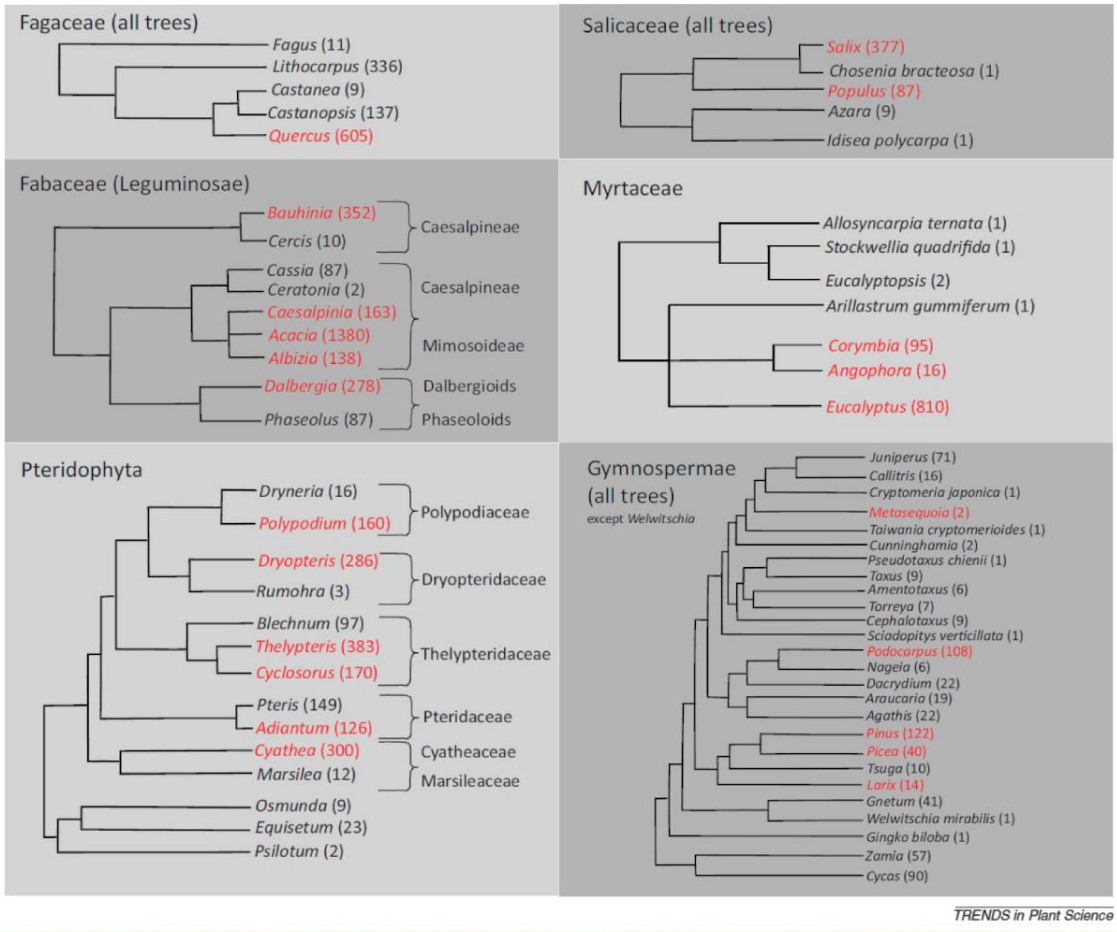

Figure I. Simplified phylogenetic trees of pteridophytes, gymnosperms, and selected arborescent angiosperm families. Isoprene emitters are identified in red.

large multimeric complexes (unlike Rubisco, for example) reflecting the scope for flexibility in gene dosage and stoichiometry [47]. Acquisition of novel functions by a duplicate gene is more likely in secondary pathways than by a gene encoding core metabolic pathways (e.g., glycolysis) [48]. This is corroborated by the fact that isoprene and monoterpene synthases, by virtue of catalysing a branch reaction in the MEP pathway, evolve faster than an average core enzyme [49]. Isoprene synthase converts dimethylallyl pyrophosphate (DMAPP) to isoprene at one of the terminal branch points within the MEP pathway. This positional hierarchy arguably adds to the plasticity of isoprene synthase amino acid identities, which are not as conserved across plants as some of the other enzymes in the MEP pathway [42,50]. Evolution of plant terpene synthases (Figure 1) is characterised by repeated loss

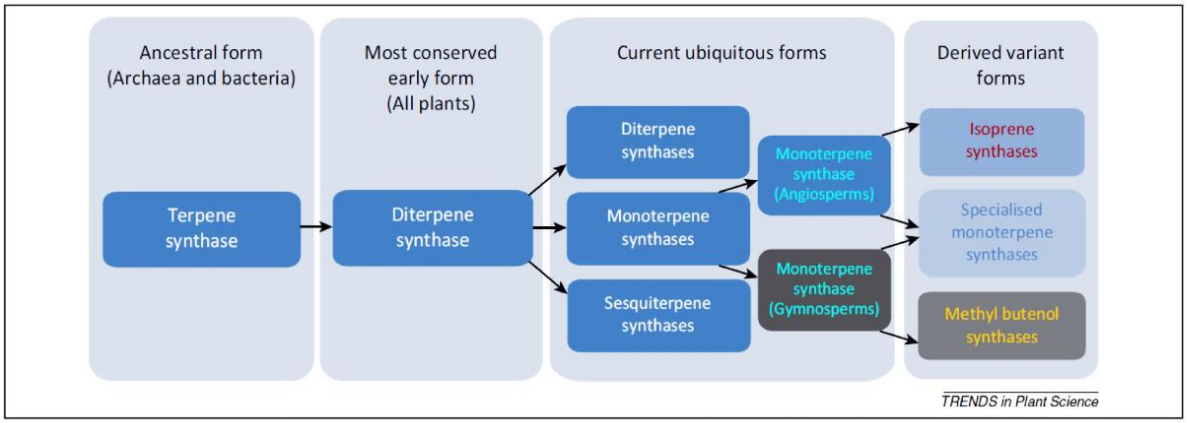

Figure 1. A schematic representation of the evolutionary history of plant terpene synthases [41,43].

and gain of protein functional domains [51]. This is consistent with the notion that isoprene synthase (and, thus, isoprene-emitting capacity) arose multiple times among plant lineages, perhaps from a malfunctioning monoterpene synthase (e.g., [52]). Some coniferous genera, such as *Pinus* and *Picea*, have acquired 3-methyl-2-buten-1-ol (MBO) synthase, an isoprene synthase analogue [7]. Notably, these MBO-emitting conifers have the highest relative diversification rates within gymnosperms (Box 1). Tree genera characterised by extensive speciation (e.g., *Ficus* spp) and hybridisation (e.g., *Eucalyptus* spp) also exhibit repeated gain and loss of isoprene emission capacity. The apparent haphazard occurrence of isoprene emission capacity among pteridophyte lineages [53] could also be explained by a similar mechanism where speciation is characterised by multiple genome duplications. More than 95% of nearly 13 000 living fern species belong to the order Pteropsida and their evolution is marked with repeated polyploidisation, a feature common to the radiation in some legumes and conifers [54].

Photosynthetic rate as a proxy for growth rate can identify isoprene emitters only among woody angiosperms (Figure 2A) and not in other plant groups, including gymnosperms, because variation in growth rate across plant lineages is a function of many factors, including nutrient availability, nutrient allocation, and longevity [55]. Gymnosperms (318 ± 79 yr) live twice as long as angiosperms (139 ± 52 yr) [56]. Thus, longevity makes up for slower growth rate among gymnosperms in increasing the number of premeiotic somatic mutations per generation. However, neither ferns nor mosses (some among both emit isoprene; [22]) exhibit fast growth rates; neither do they live as long as woody higher plants. Therefore, these lower plants appear to have evolved under genetic and physiological circumstances very different to those of spermatophytes. The following qualifications may help explain isoprene emission in lower plants: (i) the hydric habit of most isoprene-emitting plants (ferns and mosses included) is a notable feature that sits in contrast with xerophytic habit among nonemitting primitive pteridophytes (e.g., *Psilotum nudum*) and thalloid bryophytes (e.g., *Marchantia thaliana*); (ii) a perennial haploid gametophytic phase in mosses sits in contrast with a perennial diploid sporophytic phase in higher plants. Gametes in mosses are generated in a haploid unisexual gametophyte through mitosis (not meiosis) and any key mutation will be dominant because there is only one genome copy per cell. As a result, mosses, which can respond genetically to quick environmental changes [57], could also experience reduced frequency of fixation of adaptive mutations [58]. Thus, the haploid phase could lead to sporadic loss or gain of isoprene emission capacity; (iii) mosses (>6000 species) are highly polyphyletic and underwent a rapid radiation during their early evolutionary history [59,60]. The overall rates of molecular evolution in some housekeeping genes may be slower in mosses than in angiosperms (e.g., [61]) and this could have evolved as a protective mechanism to cope with the susceptibility of haploid genome to deleterious mutations. It is important to note that other genes and loci within the genomes of mosses and ferns could evolve faster than their angiosperm counterparts [61], again highlighting the relevance of gene and metabolic pathway specific plasticity.

Explaining quantitative diversity in isoprene emission levels

The observed differences in the magnitude of isoprene and monoterpene emission rates across two major emitting plant genera (gymnosperms and angiosperms) could be visualised as a function of photosynthetic rate, leaf life span, and the capacity to both store and constitutively emit monoterpenes (Figure 2B). Almost all eucalypts and evergreen conifers with long leaf or needle life spans store monoterpenes. The negative correlation between emission levels and leaf life span and the trade-off between isoprene and monoterpenes [21] supports the idea that monoterpene storing and emission have strategic advantages in evergreen habits (plant defence). Monoterpenes are stable and storable and, thus, give higher and longer-term returns per carbon share invested. High isoprene emitting genera (e.g., poplars and oaks) are deciduous trees with seasonal turnover of biomass. Deciduousness is a rare trait among gymnosperms, and it is noteworthy that almost all

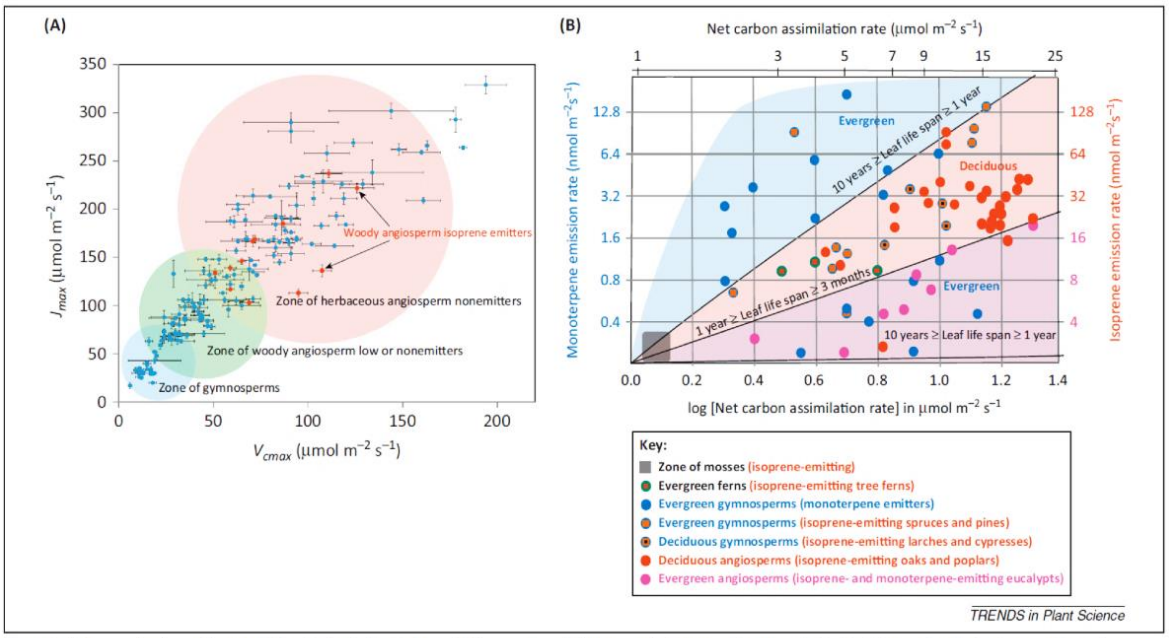

Figure 2. Photosynthesis and isoprenoid emission in land plants. **(A)** Isoprene-emitting perennials have a photosynthetic physiology of annuals. For woody angiosperms, average V_{cmax} is 47 ± 33 μmol m^{-2} s^{-1} and average J_{max} is 104 ± 64 μmol m^{-2} s^{-1}, indicated by green circular area in the middle. The smallest blue circle encompasses the gymnosperms (V_{cmax} = 25 ± 12 μmol m^{-2} s^{-1}; J_{max} = 40 ± 32 μmol m^{-2} s^{-1}). The photosynthetic capacity (a proxy for growth rate) of representative isoprene-emitting angiosperm tree genera (filled red circles) are some of the highest observed among woody plants. Their photosynthetic capacities fall within the range seen for some of the fastest-growing herbaceous annuals, including crops such as rice and wheat (indicated by translucent pale red area). The large number of premeiotic somatic cell divisions achieved through either fast growth rate (due to high metabolic rate) and/or extraordinary life spans in long-lived genera has many evolutionary consequences, one of which is the repeated acquisition and loss of isoprene emission capacity. V_{cmax} and J_{max} data from [75]. **(B)** A mosaic that relates net carbon assimilation rate, leaf life span, and isoprenoid emission rates in major plant groups. The data points correspond to carbon assimilation rates (μmol m^{-2} s^{-1}) and isoprenoid emission rates (nmol m^{-2} s^{-1}) in angiosperms and gymnosperms collated from published literature (for data and original references, see Tables S1–S3 in the supplementary material online). For all gymnosperms including isoprene emitters, use the primary y-axis (blue). For angiosperms, use the secondary y-axis (pink and red). Gymnosperms, with net assimilation rates (area basis) comparable to those of angiosperms, emit monoterpenes (and isoprene) at a lower rate than angiosperms. This could be possibly due to a relatively larger photorespiratory sink in gymnosperms (oxygen-driven sink for energy and reducing power). It is noteworthy that evergreen gymnosperms and evergreen ferns that emit isoprene fall in the regression zone of deciduous trees. For gymnosperms, net assimilation rate (A_{sat}) and emission rates of any one species were generally not derived from a single study (except a few), (see Table S2 in the supplementary material online). Therefore, the relation between assimilation rate and isoprenoid emission rate in gymnosperms should be treated only as indicative.

deciduous gymnosperms are isoprene emitters (Figure 2B). Similarly, poikilohydry and desiccation tolerance in mosses (some emit isoprene) are analogous to deciduousness in trees. The general occurrence of high isoprene emission capacity in deciduous trees makes sense for the following reasons: (i) the possibility that the deciduous habit evolved from tropical trees in their process of adapting to drier climates of high latitudes [62]; (ii) positive correlation between water-use efficiency and isoprene emission [63,64]; (iii) consistent association between moist (mesic and/or hydric) plant habitats and the isoprene emission trait [65]; and (iv) experiments suggesting isoprene emission to be of greater consequence to deciduous trees in a warming climate [66].

Evolution of plant isoprene emission capacity: driven by phylogeny or climate?

Multiple genetic origins and variants of alternative (non-C_3) photosynthetic systems in terrestrial plants are argued to be the result of both climate-driven selective pressures and the inheritance of pre-existing diverse ancestral traits [67]. By analogy, the trait of isoprenoid emission in terrestrial plants is believed to have evolved several times [68], presumably under varied abiotic selection pressures [64].

The share of carbon fixed in chloroplasts lost as isoprene is small (approximately 2%) and decreases with increasing abiotic stress because cytosolic carbon provides an alternative pool of carbon skeletons. Although long-term effects of elevated CO_2 on isoprene emission are inconclusive [9,63], it is argued that isoprene emission capacity was under either positive selection ([69]; greater value in imparting thermotolerance) or negative selection ([50]; increased carbon cost due to low photosynthesis) during low-CO_2 phases of the Phanerozoic. Monoterpene emission capacity in gymnosperms was not lost during the high CO_2 atmospheres of the Mesozoic (CO_2 >1500 ppm) [70], perhaps because a lowered V_{cmax} in gymnosperms altered the equilibrium between their energy source (photosystems) and sinks. A cumulative interactive effect of CO_2, drought, plant water-use efficiency [71,72], and the relative energy and reducing power budget (proportional to those reserved for carbon fixation) had an important role in sustaining the capacity to emit isoprene whenever the trait appeared.

Rapid diversification of angiosperms since the Cenozoic [70,72] does not explain the sporadic occurrence and

Box 2. A dichotomous key to identify qualitative and quantitative differences in biogenic isoprenoid emissions in trees

Constitutive isoprenoid emission capacity of a tree genus could essentially be identified and categorised as (low versus high) based on the following three fundamental hierarchical indicators (Figure I): (i) photosynthetic capacity; (ii) species richness; and (iii) evergreen versus deciduous habit. If the status of isoprene synthase is unknown (as is the case for most genera), then knowing the status of any one of the other three criteria will tell us whether there is a *prima facie* case for selecting a tree species for further characterisation. A broad classification of emitter class could be as follows: low monoterpene emitter (0.05–1 nmol m^{-2} s^{-1}); high monoterpene emitter (>1 nmol m^{-2} s^{-1}); low isoprene emitter (0.05–10 nmol m^{-2} s^{-1}); or high isoprene emitter (>10 nmol m^{-2} s^{-1}).

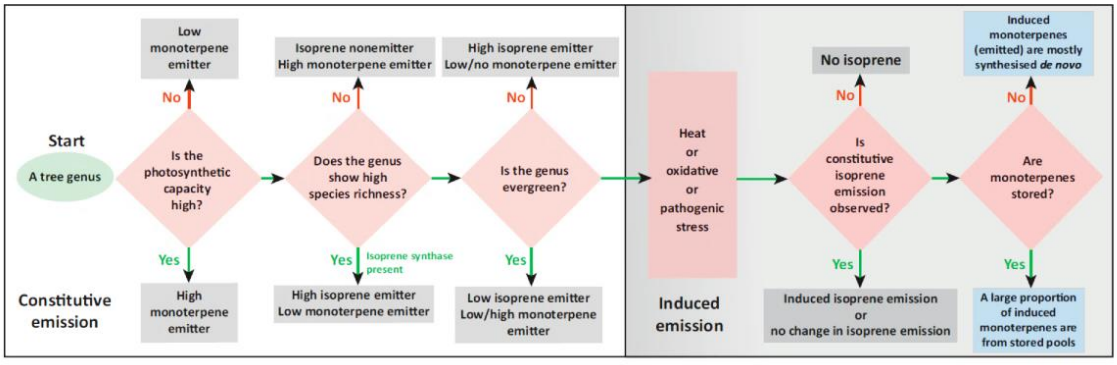

Figure I. Identification of the isoprenoid emission trait of tree genera.

quantitative diversity in isoprene emission across plant clades. Although gene pool isolation and patterns of inheritance had a role [73], the trait also owes its occurrence to the 'high-octane' physiology of some perennial plant genera. More fundamentally, isoprene emission is not a universal mechanism that could arise in all plants in response to a single or combination of environmental factors (be it CO_2 and/or temperature).

Concluding remarks

There have been many laudable attempts to trace the phylogenetic history of isoprenoid emission trait among extant plant taxa (e.g., [50,53,74]), yet a larger underlying pattern has been hard to establish. Qualitative and quantitative diversity in isoprenoid emissions is partly explained by photosynthetic rates seen in conjunction with the broad spectrum of leaf economics (Box 2) [21]. We hypothesise that traits such as high growth rates, high speciation rates, long generation times, and isoprene emission capacity seem to coevolve without phylogenetic affiliations. This can also explain why constitutive isoprene emission has been repeatedly gained and lost, resulting in the disjunct distribution of the trait across unrelated plant lineages.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tplants.2014.01.009.

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Chapter 7

Biogenic volatile isoprenoid emission and levels of natural selection[7]

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REVIEWS

Biogenic Volatile Isoprenoid Emission and Levels of Natural Selection

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Abstract | Biogenic volatile isoprenoid emission as a biological process has many worthwhile yet unanswered questions of fundamental scientific and ecological merit. Foremost among them is to understand and quantify the long-term feedback effects of volatile emission on climate and climate-driven macro-evolutionary changes. Moreover, we are now at a stage where our understanding of biogenic isoprenoid emission at the molecular and ecophysiological levels holds the key to the doors of next generation breakthroughs in isoprenoid-dependent applications in synthetic chemistry, human bio-therapeutics and agro-food industries. Like any other living trait/process, biogenic volatile isoprenoid emission has several levels of complex organization. We summarise biophysical, chemical and ecological functions of biogenic volatile isoprenoid emission highlighting aspects of evolution at different levels of natural selection.

Keywords: *ecological fitness, evolution, isoprene, isoprenoid biosynthesis, natural selection, photosynthesis, volatile organic compounds*

1 Introduction

Constitutive volatile isoprenoid emission by phototrophic living organisms is a process whose biological costs are not trivial while evidence of the (possibly multiple) benefits are still circumstantial, or purely elusive. More than 1000 Tg carbon per year is emitted in the form of volatile isoprenoids, mainly isoprene (C_5H_8) and monoterpenes ($C_{10}H_{16}$), mainly by terrestrial plants[1] and, as far as we currently know in much lower amount (~10 TgC/yr) by marine phytoplankton.[2,3] To put this in perspective, this is comparable to the carbon loss caused by global deforestation[4] (~1200 TgC/yr). The emitted isoprenoids have a prolonged post-emission impact on the climate, especially through oxidation chemistry of ozone in the troposphere, and formation of secondary organic aerosol and precipitation.[5–7]

Isoprenoids (also called terpenoids) are a large class of versatile macromolecules with great structural diversity despite being all constructed by catenation of five carbon (C5) monomers that are derivatives of isopentenyl pyrophosphate (IPP) and dimethyallyl pyrophosphate (DMAPP). Volatile isoprenoids are small terpenoids whose conjugated double bounds (dienes) readily react with any atom/molecule with unpaired valence electrons. These are made by one (isoprene), two (monoterpenes) or three (sesquiterpenes) C5 units. Drawing from recent research developments, in this review we examine the phenomenon of biogenic volatile isoprenoid emission from an evolutionary standpoint, at different levels of organization spanning a single living prokaryotic cell to populations of forest trees (Fig. 1, Box 1).

2 A Flexible Structure to Function Relation Among Isoprenoids has Accommodated Long Periods of Neutral Drift in Molecular Evolution

The diversity in isoprenoid emission capacity is a product of interactions between many genes, enzymes and metabolites both within and across interacting pathways. The enzymes involved in isoprenoid biosynthesis belong to a family of closely related terpene synthases (TPSs).[8] Since there is no significant homology between plant TPS sequences and the known bacterial genomic equivalents, it is inferred that bacterial and plant TPSs do not share a common ancestry[9] although

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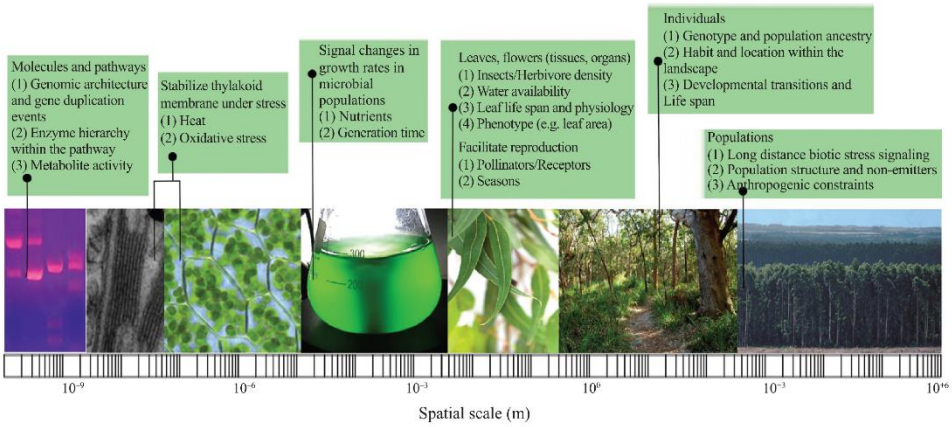

Figure 1: Function of volatile isoprenoids (isoprene and monoterpenes) and means of natural selection.

Box 1 Levels of Natural Selection and the Isoprenoid Emission Trait

Underneath any biological phenomenon, there lies a hierarchy of organization and levels of natural selection that shape and reshape its evolution at different levels from molecules to tissues and from organisms to populations/ecosystems. The debates about the actual unit of natural selection, from the unit being a single gene to a group of organisms, have continued to generate fascinating evolutionary enquiries and polarised disagreements.[96–98] If there is one thing that is becoming clear then it is the modular nature of the unit of selection. By modularity we mean either a hierarchical or egalitarian network of interacting elements.[99,100] A module could be a group of genes (elements) co-expressed/co-regulated by similar environmental stimuli. A module could also be a higher order organization that defines tissues and/or organisms. As life organizes itself into intricate interactions, it is of great benefit to strip any trait/behaviour/process of interest into its fundamental components and dissect discrete facets of natural selection at each level of modular organization.

Level 1: Genes (and enzymes) are seen as the site of natural selection in continuous action since genes are the simplest self-replicating and perpetual entities unlike their carrier organisms which undergo life and death[101,102] (see section 2, Tables 1 and 2).

Level 2: The cell provides a physical framework that facilitates self-correction and selective regulation of a large self-replicating and error-prone chemical regulatory system. Natural selection finds its way through a complex set of cell organelles that share space and resources. The cell membrane concentrates the medium of life within a cell and allows biochemical reactions that are 10 times faster than those in cell-free systems[103] (see sections 3 and 4).

Level 3: Phenotypic characters such as phyllotaxis, leaf shape and area, canopy architecture, etc., are all products of life history evolution and 'canalization', a process in which phenotypes are locked by genotypes and become potentially insensitive to environmental stimuli.[104] Deviation from such set norms constitutes phenotypic plasticity, which is a raw material for natural selection (with or without heritability) especially in long-lived plants[105,106] (see section 5).

Level 4: When phenotypic variation within a population, with regard to a specific trait or a group of traits, is heritable and its occurrence follows the spatial genetic structure of the population, then such traits of one individual will have differential fitness effects on neighbouring individuals[107] (see section 6).

some enzymes are proposed to be monophyletic at least within major plant clades.[8,10] Deduced amino acid sequences of large TPS gene families in some gymnosperms and comparative analyses of angiosperm TPSs suggest that modern TPSs could have evolved from an ancestral diterpene synthase in a eukaryotic ancestor of higher plants, much before specialization of TPS functions and divergence of angiosperms and gymnosperms could take place.[11,12]

Divergence: acquisition of dissimilar characters or traits by related organisms.

Despite an apparently simple chemical structure, a very large number of volatile isoprenoids are present in nature. The idea of "molecular parsimony" suggests that large populations of chemical metabolites (e.g. >60000 terpenoids) with minor differences are synthesized by a relatively small group of enzymes (per organism) because (a) the probability of hitting a chemical conformation of significant potency is always small,[13] (b) the cost of gene transcription and RNA translation could influence organismal fitness,[14] and (c) promiscuity is the norm in enzyme evolution irrespective of enzyme–substrate specificity.[15] TPSs are known to be promiscuous in that they can act on different versions of related substrates and thus may have been the main contributors to the vast diversity of isoprenoids.[16] Moreover, significant single nucleotide polymorphisms (SNPs) in genes encoding for TPSs are associated with the qualitative and quantitative variation in isoprenoid emission profiles.[17]

Minor changes in chemical structures involved in physical/structural function (e.g. membrane lipids, accessory pigments) do not necessarily compromise their functions, and thus are rarely constrained by stringent natural selection.[18] As a result, the same metabolite (e.g. ascorbate[19]), with or without minor changes, could acquire novel biochemical functions under different circumstances across divergent clades. The idea of 'superior biomolecular activity'[20] is proposed to explain structural conservatism and functional divergence in chemical molecules. Often, natural selection promotes 'a chemical blend' (of various stored aromatics and monoterpenes) rather than a specific structural configuration of a single volatile isoprenoid and such chemical mixtures are more potent than a single molecule in terms of 'biomolecular activity'.[21] In this way certain monoterpene blends increase beneficial biological interactions and in some cases are stringently selected to suit interactants in co-evolved biological systems involving flower–pollinator, host–pathogen and plant–herbivore interactions[22] (also see section 5).

3 Modes, Mechanisms and Energy Budget of Carbon Reduction Could be the Major Factors Determining Emission Potential of Isoprenoids

Independent prokaryotic origins of plant cell organelles have resulted in multiple biosynthetic pathways with duplicated functions (*sensu lato*) taking place simultaneously within different organelles.[23,24] Plant isoprenoid biosynthesis occurs through one of the two spatially separated pathways within a plant cell (Fig. 2). The cyanobacterial pathway takes place in the plastid, and is also referred to as the methyl erythritol phosphate (MEP) pathway; while the archaeal pathway, operating in the cytoplasm, is also referred to as the MVA (mevalonic acid) pathway.[25–27] The MVA pathway proceeds further into multiple terminal pathways including steroid and hormone biosynthesis. The MEP pathway proceeds further to synthesise stable and structural terpenoids such as carotenoids.

Several models have been proposed to explain the evolutionary events that caused the divergence of archaea and bacteria; however, there is no consensus.[28] Sequence similarity and pathway reconstruction analyses show that at least three separate horizontal 'whole pathway' transfer events between bacteria and archaea could have taken place[29] but the nature of either the original eukaryotic ancestor or the origin of isoprenoid-dependent membrane architecture remains unexplained.[30] Both MEP and MVA pathways are almost mutually exclusive among prokaryotes with the exception of *Streptomyces* species, which possess both pathways and it is hypothesized that maintaining both pathways could be beneficial for the bacterium since it allows selective use of resources and specialized functions.[31,32] It is not clear which pathway appeared first.

Mutual exclusivity of the two pathways also holds among some eukaryotes such as fungi and animals that possess only the MVA pathway.[33] But, the simultaneous occurrence of the two pathways in plants, despite organellar (spatial) separation, creates a complex scenario for natural selection to act on the functionality of this system.[24] In fact, (a) all the genomic controls on both the pathways are in the nucleus (the MEP pathway enzymes are nuclear encoded and plastid targeted), and (b) the substrate level cross talk between the two pathways takes place through a selective chloroplast membrane interface through selective transport of IPP from plastid to cytosol.[34] The transport of glyceraldehyde-3-phosphate (GAP) from plastid to cytosol, and of phospho-enol-pyruvate (PEP) from cytosol to plastid, suggests 'an umbilical link' between the two pathways, as first postulated[35] and later confirmed through labelling experiments.[36] It was demonstrated that the genetic blocking of either the MVA pathway or the MEP pathway in null mutants, or the complete inhibition of single pathway enzymes in wild-type plants treated with specific inhibitors resulted in a developmental block and a seedling-lethal phenotype. This indicates that the loss of one of the two pathways cannot be compensated by the remaining pathway.[37]

Parsimony: the minimum number of evolutionary changes to infer phylogenetic relationships between closely related taxa. Parsimony also implies that the simplest hypothesis (among many alternatives) that is sufficient to explain an observation is to be preferred and is most likely to be closest to the reality.

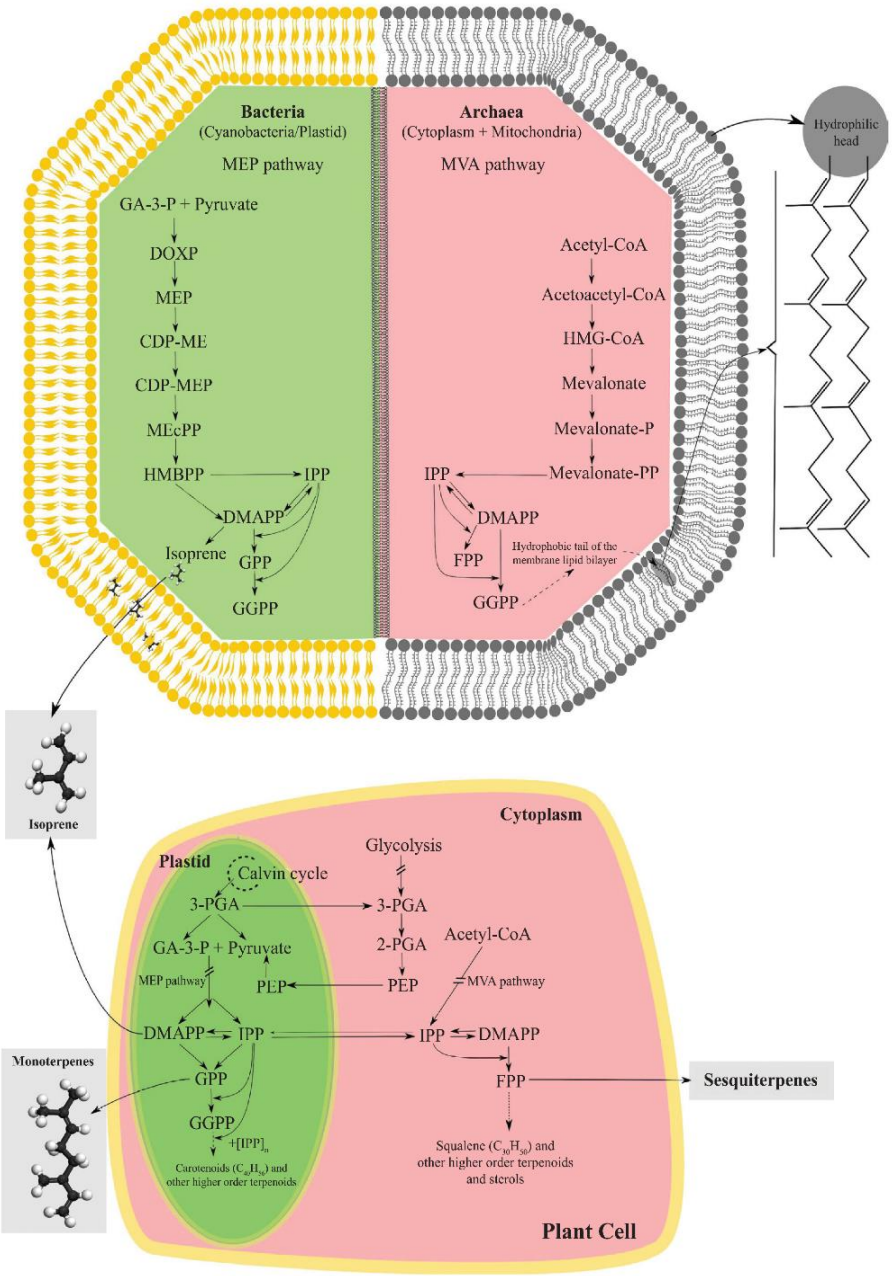

Figure 2: Isoprenoid biosynthetic pathways in bacteria and archaea (top) and in plants (bottom).

Although the MEP pathway contributes to volatile isoprenoid emission in eubacteria as well as in higher plants, surprisingly the MVA pathway outperforms the MEP pathway in *E. coli* engineered for large scale production of isoprenoids.[38] Metabolic flux through the MEP pathway has several bottlenecks and involves allosteric feedback mechanisms.[39,40] The MEP pathway gene expression is regulated by heat, light and circadian transcriptional factors,[41,42] which appear to follow hierarchical and modular organization.[43] The difficulty of working with the MEP pathway is

due to our lack of understanding of the pathway's evolutionary history. Knowing the two pathways and the selection pressures acting on them (e.g. with reference to gene specific subtleties in codon usage, and oxidative stress sensitivity) will provide important leads in solving problems, especially for large scale microbial production of industrially relevant isoprenoids.

The reasons for differences in productivity and efficiency of these pathways in heterologous expression systems potentially lie in distinct and ancient evolutionary histories of bacteria and archaea, and their ways of acquiring and reducing carbon. While photosynthesis by photoautotrophs (starting with cyanobacteria) is the most influential biological phenomenon in the history of the Earth, it is neither the most ancient nor the most efficient way through which carbon could be reduced for storage and transport. Alternative autotrophic carbon reduction pathways had evolved much before photosynthesis among early prokaryotes, mostly extremophiles.[43] Among those, the chemoautotrophic reduction of carbon to acetyl-CoA is a prominent process that supplies carbon to the MVA pathway in archaea and some bacteria. Comparison between energy demands of alternative autotrophic carbon reduction pathways among extant prokaryotes clearly shows large differences in costs per fixed carbon.[43,44] The Rubisco-based aerobic CO_2 fixing system is the most expensive among all known autotrophic CO_2-fixing mechanisms. Correspondingly, photoautotrophs have a large energy and reducing power capacity that not only sustains expensive high turn-over of enzymes and their maintenance but also supports constitutive volatile isoprenoid emission via the MEP pathway which also involves several steps of chemical reduction.[45] It is known

Box 2 Codon Bias and Amino Acid Usage: Contrasting MEP and MVA Pathways in Angiosperms

Table 1: Comparing amino acid frequency in enzymes of two spatially separated isoprenoid biosynthetic pathways in plants.

	Frequency of amino acid per 1000 residues/enzyme‡		
Amino acid	MEP pathway (*N* = 7 enzymes)* mean ± 1 SE	MVA pathway (*N* = 6 enzymes)** mean ± 1 SE	*P* value ($\alpha = 0.05$) *t* test
Ala	76 ± 6.9	92 ± 8.8	0.165
Cys	**15 ± 0.6**	**21 ± 0.7**	**0.000**
Asp	59 ± 2.0	46 ± 6.1	0.094
Glu	60 ± 5.4	57 ± 3.4	0.687
Phe	39 ± 4.2	35 ± 2.9	0.514
Gly	72 ± 4.7	77 ± 8.1	0.601
His	25 ± 4.5	20 ± 2.2	0.377
Ile	60 ± 2.5	55 ± 2.8	0.197
Lys	65 ± 3.7	58 ± 3.8	0.195
Leu	95 ± 6.2	100 ± 9.7	0.677
Met	20 ± 2.9	26 ± 2.3	0.159
Asn	35 ± 3.7	42 ± 2.0	0.143
Pro	**55 ± 5.0**	**43 ± 2.9**	**0.059**
Gln	31 ± 3.1	33 ± 2.0	0.691
Arg	46 ± 4.0	40 ± 3.1	0.296
Ser	85 ± 11.0	88 ± 3.6	0.821
Thr	51 ± 2.2	50 ± 3.0	0.811
Val	73 ± 4.2	74 ± 4.8	0.912
Trp	9 ± 1.9	11 ± 2.7	0.533
Tyr	26 ± 2.7	26 ± 6.4	0.949
TOTAL†	998	995	

**DXS, DXR, MCT, CMK, MDS, HDS, and HDR; **ACCT, HMGS, HMGR, MVK, PMVK, and PMVDC;* †Total excludes stop codons (For enzyme names, see Table 2); ‡the enzymes responsible for reactions up to the formation of IPP/DMAPP.

Box 2 Continued

Codon bias: the propensity to use a particular triplet codon to specify a particular amino acid.

Codon bias towards optimal/common codons positively correlates with gene expression levels. Abundance of tRNAs influences translational efficiency.[108–110] Codons coding for rare and structurally critical amino acids possess the least abundant tRNAs, viz. cysteine, tyrosine and tryptophan, which curiously also have the highest probability of mutating into stop codons given the way triplet codons have evolved. Mutations that replace less abundant and structurally important amino acids are always minimised and as a result premature terminal mutations are also minimised by natural selection. Codon usage frequency, calculated for each enzyme in both MVA and MEP pathway using a set of at least three representative sequences from angiosperms of which one sequence within each set was from *Arabidopsis thaliana*, showed no significant difference in overall codon frequency (relative to genome wide codon usage in *Arabidopsis*) between all the genes between and within both pathways with minor exceptions. However, the differences became significant when the pathways were divided into two sections with the top section resulting in IPP/DMAPP biosynthesis and the bottom section involving terpenoid synthases and prenyltransferases. The top section of the MVA pathway comprised significantly more cysteine than the MEP pathway (Table 1). Given adverse factors such as (a) limited availability of $tRNA^{Cys}$, which is among the least abundant tRNAs in plants[111] and (b) oxygen sensitivity of the thiol group that puts negative selection pressure on cysteines in cytosolic proteins,[112] cysteine richness of MVA pathway enzymes in plants must have ancient phylogenetic constraints. Cysteine richness in the MVA pathway enzymes is perhaps consistent with the pathway's archaeal evolutionary ancestry given that enzymes in thermophilic archaea were rich in disulfide bonds and were selected to remain stable under extreme temperatures.[113] It is also not a coincidence that energetically less expensive (relative to the Calvin cycle) chemoautotrophic CO_2 fixing mechanisms are restricted to anaerobic habitats (e.g. sulphur bacteria and methanogenic archaea; also see section 3), where cysteine richness in MVA pathway was not under negative selection.

Table 2: Genes and enzymes of the MEP and MVA isoprenoid biosynthetic pathways in plants.

Gene	Corresponding enzyme of the MEP pathway	Gene	Corresponding enzyme of the MVA pathway
Top section of the two pathways (reactions leading to the formation of IPP/DMAPP)			
DXS	1-deoxy-D-xylulose 5-phosphate synthase	*ACCT*	Acetyl-CoA C-acetyltransferase
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase (aka: CM synthase)	*HMGS*	3-Hydroxy-3-methylglutaryl-CoA synthase
MCT	2-C-methyl-D-erythritol 4-phosphate cytidylyl transferase	*HMGR*	3-Hydroxy-3-methylglutaryl-CoA reductase
CMK	4-diphosphocytidyl-2-C-methylerythritol kinase	*MVK*	Mevalonic acid kinase
MDS	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	*PMVK*	Phospho-MVA kinase
HDS	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase	*PMVDC*	Diphospho-MVA decarboxylase
HDR	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase		
Bottom section of the two pathways (reactions leading to the formation of isoprenoids)			
IPI	Isopentenyl diphosphate isomerase		
ISPS	Isoprene synthase	NA	NA
GPS	Geranyl pyrophosphate synthase		
MTS	Monoterpene synthase	NA	NA
GPPS	Geranyl geranyl pyrophosphate synthase		
PS	Phytoene synthase	NA	NA

that both isoprene and monoterpene biosynthesis through the MEP pathway principally utilises carbon fixed *de novo* during photosynthesis.[46–48] The carbon cost of volatile biosynthesis in plants is around 2% of photosynthesis under stress-free conditions, and increases under abiotic stress, often exceeding 10% of fixed carbon[49] (both *de novo* and imported from cytosol) with a corresponding increase in energy costs.[50–52]

Atmospheric CO_2 concentration is a key regulator of photosynthesis and it is also shown to influence volatile isoprenoid emission. Increasing CO_2 concentration increases photosynthesis and decreases isoprene emission, at least during short-term acclimation,[53–54] but also in plants exposed to life-long high CO_2.[55] Many have argued a case for CO_2-driven emission changes through geological history.[56] The high cost on a plant's carbon budget due to emission especially during low CO_2 eras (glaciations), in turn likely constraining photosynthesis, may have exerted a significant negative selection pressure on emission capacity.[10] The same logic does not apply to archaea and the MVA pathway, since none of the known autotrophic archaea employs the Calvin cycle to fix carbon. However, the fact that low CO_2 causes increased emission in plants at least over short-term acclimation contradicts the notion of negative selection on emission during glacial periods. The suggestion is confounded by the complex interactive effects of heat and CO_2 on emission, which do not fit any existing mechanistic theories of emission behaviour.[57] There is increasing experimental evidence in favour of a hypothesis proposed in the 1990s that availability of energy (ATPs) controls isoprene synthesis.[58] ATP and reducing power (NADPHs and equivalents) unused by carbon reduction may explain isoprenoid emission behaviour under varied CO_2 and drought scenarios.[52,59–61] As put forth earlier, these findings further support the idea that the mechanisms and energy budget of carbon reduction could have been the primary factors through which natural selection determined isoprenoid emission capacity and behaviour in prokaryotes and later in plants (also see Box 2).

4 Isoprene Emission is Sensitive to Abiotic Stress Operating at the Level of a Single Cell and Population of Cells

Isoprene emission has distinct and testable physical functions at the level of cells (prokaryotes and unicellular eukaryotes) and multicellular tissues. Hypotheses concerning isoprene-mediated scavenging of free radicals[62,63] along with possible membrane stabilization under transient heat stress in higher plants[64] have strong empirical evidence, and now also some mechanistic understanding.[65–67] In thermophilic archaea, isoprenoid-linked phospholipids form their cell membranes (Fig. 2), while in bacteria emitted isoprene could simply physically interact with bacterial membrane as proposed in eukaryotes. However, the function of spurts in isoprene emission at different growth phases among some bacteria is still unknown.[9] The function of isoprene emission among marine phytoplankton such as dinoflagellates and diatoms is also unclear.

Natural selection influences the inter- and intra-specific variation in any heritable characteristic, and the extent of genetic differentiation in certain loci in the genome may reveal the nature of selection.[68] As mentioned earlier, isoprenoid synthases are promiscuous enzymes with flexible substrate affinities and often evolve through gene duplication events. The newly acquired functions of a duplicated enzyme are normally not efficient and it is hypothesized that hyper-transcription could overcome enzymatic inefficiencies in secondary metabolism.[69] A gene duplication event could often be followed by selectively neutral mutations in the duplicate gene; thus neofunctionalization is rare and gene redundancy is common.[70] Therefore, the random emergence of isoprene synthase only in certain limited number of plant phylogenetic clades with altered specificity/efficiency remains unexplained. Two alternative theories have been proposed. One of them posits that the occurrence of isoprene emission capacity in unrelated land plant lineages may be explained by specific environmental conditions that increase fitness of emitting species.[71] In an experiment involving isoprene emitting and non-emitting (RNA*i* mutant) plants acclimated to glacial CO_2 levels (190 ppm), it was found that photosynthesis recovered faster in isoprene emitters than non-emitters after a heat or sun-fleck stress treatment[72] suggesting that low CO_2 periods during the Quarternary could have positively selected for isoprene emission capacity resulting in high emitting plants. However, in most cases, the evolutionary advantage to the isoprene emitting genera is unclear because the trait is often randomly lost. Even if we assume that isoprene emission confers additional fitness to the emitting genus,[73] the fact that isoprene synthases have remained very inefficient (K_m > 2 mM) compared to other enzymes of the MEP pathway suggests weak selection.[74] To explain random disappearance and reappearance of isoprene emission in certain limited number of plant lineages we should

Calvin cycle: also known as the photosynthetic carbon reduction cycle. A cyclic pathway used for the fixation of carbon dioxide by photoautotrophic organisms.

Mechanistic theory and modelling: when natural processes are mechanistically determined and the laws of physics and chemistry are sufficient to explain biological phenomena. Mechanistic modelling involves deduction of mathematical relationships between (often) biological variables and it emphasizes the physical, chemical and biochemical principles that explain the observed relationship between variables.

Natural selection: the non-random and differential reproduction of different genotypes acting to preserve favourable variants and to eliminate less favourable variants.

Fitness: the relative competitive ability of a given genotype conferred by adaptive morphological, physiological and behavioural characters. Fitness is often quantified as the average number of surviving progeny of one genotype relative to that of competing genotypes.

Quarternary: period of geological time that covers approximately the last 1.8 million years. The Quarternary is noted for numerous major glacial-interglacial cycles (advancement and receding of polar ice sheets) characterized by significant shifts in global temperature and atmospheric CO_2 concentration.

also account for the roles played by fast growing physiology and long life span in isoprene emitting trees in accumulation of pre-meiotic mutations and tolerance for such mutations in the corresponding loci of their genome.[75]

5 Monoterpenes and Other Aromatics are Under Stringent Selection in Co-Evolved Biological Systems

Plant behaviour, especially in annuals, does not evolve during the course of a plant's lifetime because plants do not have memory in the sense of experience storage within neuronal connections as in animals. Plants "rote-learn" through evolution by natural selection, a very slow process usually operating over many generations at the level of genes, genomes, epigenomes and possibly phenotypic plasticity (which may or may not be determined by genotypes). As a result, several traits may persist in plant populations long after they have lost adaptive relevance. While this might arguably be the case for isoprene emission in long-lived trees (but see[64]), other volatile isoprenoids have well-defined roles in plant defence. For example, induced volatile isoprenoids could be employed effectively to prime intraplant antiherbivory responses.[76–78] Many metabolites derived from the MVA pathway, primarily sesqui-, di-, and saponin tri-terpenoids, have potent antifungal, antimicrobial, and repellent properties or serve to attract predators or parasitoids.[79]

Rote-learning: learning or remembering by repetition rather than through developing an association between phenomena.

Less volatile monoterpenes can be stored under most conditions and their induced emission has an important role in maintaining organismal fitness. Constitutive emission of monoterpenes has been shown to defend plant parts that are at high risk of intense herbivore attack, preventing the loss of those parts that might result in substantial fitness costs.[21] The fact that foliar volatile storing is virtually absent in deciduous trees, which likely are vulnerable to insect attack, suggests a link between monoterpene emission and structural costs of plant parts (also see section 6).

Intraspecific volatile communication mostly involving aromatics and monoterpenes could increase population fitness by benefiting closely related individuals within a large population[80] (also see Box 1). There is evidence to suggest that interspecific signaling and recognition both in aquatic and terrestrial ecosystems involve volatile isoprenoids.[81,82] However, high-cost resource mediated communication strategies involving tertiary trophic interactions (e.g. bodyguard insects such as parasitoid wasps or ants) appear to be under more stringent selection than low-cost constitutive or induced information transmission between plant populations.[83] It remains to be seen whether untargeted, constitutive isoprenoid emission of a species is a function of ecosystem heterogeneity.[84]

Plant functional type (PFT): a collection of plant species (vegetation) with similar suits of co-occuring functional traits that exhibit similar responses to external stimuli and have similar effects on ecosystem function. The equivalent of a PFT in the animal kingdom is a '*guild*'.

6 Reconciling Phenotypic, Genetic Diversity and the Sensitivity of Emission Responses in Individuals or Populations of Trees to Environmental Stimuli

Stringent developmental constraints on isoprenoid emission levels (see review[85]) point towards natural selection acting on the whole plant phenotype. Leaf economics and leaf life span have a significant influence on isoprenoid emission profiles.[53,61] Physical defense strategies involve niche-specific phenotypic adaptations and phenotype constrains chemical defence. For example storing of volatiles is determined by packing efficiency of monoterpenoid storage glands in leaves, which in turn depends on specific leaf area, distribution and thickness of palisade parenchyma. In addition, natural selection appears not to have favoured a trade-off between chemical and physical defences in most plants,[86] which could ultimately mean that isoprenoid emission may not show any relationship with the broad trends in plant phenotypic strategies. On the whole, reconciling phenomic variability with genetic diversity and accounting for their cumulative impact on isoprenoid emission profile has not been possible despite significant progress on both fronts. The contradictions between field trials that ignore genetic variation and species-specific controlled experiments that aim to minimize environmental variation have made the challenge more complicated than it ought to be.[87]

The importance of knowing how natural selection is acting on emission behaviour of plant populations is exemplified by the challenges faced by models trying to forecast global isoprenoid emissions given the significant impact of emissions on regional climate and carbon cycles. Most isoprenoid emission models are parameterized based on leaf level isoprenoid emission measurements and there is a large uncertainty in emission variation within plant functional types (PFTs).[88] There are many local emission discrepancies that are hard to explain,[1] and generalizations are not helped by the fact that emission capacity does not follow consistent phylogenetic patterns.[72,75,89,90] Vegetation models assume presence or absence of a defined PFT, which ignores the significant impact of fine-scale genetic variations on ecosystem dynamics and their impact on spatial dimensions of emission

signature.[91] Ignoring intra-population (fine-scale) variations might work in case of homogeneous clonal plantation emitters, some of which are indeed the most dominant isoprene emitting angiosperms.[89,92] However, many monoterpene emitters in boreal forests (pines, firs, spruces and oaks) exhibit low domestication and live in large, open-pollinated, native populations. Genetic diversity, gene flow, population heterogeneity and structure (relationship between individuals belonging to a single species) is shown to affect VOC emission profiles[93] and such effects are likely magnified in species spread over large geographic areas.[94] Reassessment of recent literature on plant emission response to changing climate has led to the suggestion that carbon input in the form of isoprenoids into the climate system will increase in future.[58] Anthropic pressure to select suitable agricultural traits might also have contributed to diversify emission in cultivated plants, as found in cork oaks over their cultivation range.[94]

7 Going Forward

Simple chemical derivation (reduction), highly reactive hydrocarbon chemistry, a wide range of ecological benefits, and an unlimited scope for diverse structural configurations have contributed to repeated emergence and functional diversification of biogenic volatile isoprenoid emission in evolutionarily distant and unrelated living systems. It is helpful to remind ourselves that each step during the development of a living organism in some ways represents a cusp of one of the major transitions in the evolution of living complexity.[95] We are still aiming at discovering metabolic and biophysical aspects of isoprenoid emissions in eubacteria and protists (diatoms and dinoflagellates), and such information is likely needed to further decode the complexity of volatile emissions in higher plants. With every new finding about some aspect of metabolic regulation of isoprenoid emission, it is becoming clearer that emission from living organisms provides a template to investigate other complex biological phenomena with often unclear function and uncertain origins. At the other end of the spatial scale, the consequences of land-use (vegetation) changes and increased temperatures due to unprecedented anthropogenic interference will not only have an impact on air quality and human lifestyle in a rapidly urbanising world, but is also likely to change the isoprenoid emission profiles of emitting and non-emitting living systems.

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Chapter 8

Conclusion and future directions[8]

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***Plant Signaling and Behavior* (see Appendix VII)**

[8] **Contributions**: KGSD et al. were invited by the editor of *Plant Sigaling and Behavior* to write an *addendum* to their paper published in *Plant Physiology* (Chapter 3 of this thesis). KGSD wrote the chapter content and wrote the manuscript with corrections from BJA and IMJ.

Conclusion and future directions:

Terrestrial plant ecosystems are a major sink for CO_2 and constitute a central component of global carbon cycle. The emission of isoprene (~0.5 PgC/yr) by forests releases carbon to the atmosphere in a form that influences tropospheric oxidation reactions and volatile isoprenoids are an important natural source of secondary organic aerosol impacting cloud formation (Kavouras et al. 1998; Guenther et al. 2006). Isoprene biosynthesis and emission by plants depends on carbon, energy and reducing power supplied by photosynthesis (Delwische et al. 1993; Loreto and Sharkey, 1993; Seemann et al. 2006). The interactive effects of CO_2, heat and drought on photosynthesis and isoprenoid emission are quite complex. Emissions increase in plants under oxidative stress caused by high light, low CO_2, drought and heat; such observations have led to various hypotheses concerning the biological function and evolutionary role of isoprene emission in ecosystems (reviewed in Sharkey and Monson, 2014). In this concluding chapter, we discuss the overall significance of the published papers presented in the thesis in addition to highlighting important results from other chapters that contain unpublished data. During this discussion, we identify pertinent gaps in our knowledge and point towards future investigations that we believe will help greater understanding of plant isoprenoid emission.

Eucalypts are the most important evergreen tree taxa in the Southern hemisphere in that they harbor the most dense terrestrial carbon stores on the planet (Keith et al. 2009). Although eucalypts do not emit volatiles isoprenoids to the same extent of temperate poplars or oaks, the fact that tropical evergreen ecosystems are the world's most significant

source of isoprene (Guenther et al. 2006) make many tropical genera like eucalypts with pan global geography a significant source of isoprenoids to the earth system. Eucalypts exhibit extraordinary adaptations and offer a wide array of ecophysiological contrasts to study some of the finer unknown aspects of the relationship between photosynthesis and isoprenoid biochemistry in plants (see **Chapters 1 and 2**). A hypothesis that attempts to explain variation in isoprenoid emission states that *secondary metabolism (in general) and volatile isoprenoid emissions increase under abiotic stresses because the net carbon assimilation declines but the supply of reducing equivalents remains high* (Niinemets et al. 1999; Harrison et al. 2013; Selmar and Kleinwachther, 2013; Morfopoulos et al. 2014). In **Chapter 3** (published) of this thesis, **we provide substantial experimental evidence supported by a mechanistic model in favour of the hypothesis that photosynthetic carbon reduction competes with non-photosynthetic sinks for reducing power under stress-free conditions (Dani et al. 2014a)**. **A claim is made that residual reducing power unused by carbon assimilation drives increased isoprene emission under various abiotic stresses.** Exploiting the contrasting drought tolerance of two *Eucalyptus* species, we provide an exhaustive experimental data set showing that photosynthetic electron transport rate (ETR) remained relatively more robust under drought compared with net carbon assimilation rate (NAR). Consequently, an increased ETR-to-NAR ratio sustained increased isoprene emission rates under drought, which appears to follow a species-specific threshold for drought tolerance.

CO_2 compensation point (Γ*) is an important indicator of photorespiratory capacity of a species and it takes an appreciable range of values even among C_3 plants (30 to 70 ppm; Black, 1973). Drought causes stomatal closure to increase leaf temperature and CO_2 compensation point. Γ* also shows a diurnal cycle (40 ppm to 160 ppm) with a daily maximum coinciding with midday depression in carboxylation efficiency (Tenhunen et al.

1984). The well-known diurnal peak of isoprene emission just around after midday is consistent with the hypothesis in Chapter 3 and coincides with the midday depression in carboxylation efficiency. We have observed a large range in Γ* values (40 ppm to 80 ppm) across many species of the genus *Eucalyptus* under stress-free conditions (data not shown). The large range in Γ* could be due to diversity in leaf structural traits (mesic vs. xeric) that reflect rainfall and nutrient availability in their respective habitats. Photorespiration directly competes with the MEP pathway for reducing power and the competition becomes acute under drought stress as photorespiration rate increases and carbon supply for the MEP pathway decreases (Chapter 3). Differences between more distantly related taxa in their photorespiratory sink strengths for carbon and reducing power will make future projections of global isoprenoid emission far more difficult using existing models and mechanistic assumptions.

Isoprenoid emission rates observed in all of our experiments involving eucalypts were low (0 to 10 nmol/m^2/s) compared with those from more widely studied poplars and oaks (20 to 100 nmol/m^2/s). There are some rare exceptions in the literature (for a survey see the Lancaster isoprenoid emission inventory) but such exceptions are most likely due to measurement conditions in the field. Many native Australian plants have abundant Rubisco and eucalypts in particular are notable for their extremely high photosynthetic capacities amongst trees, operating generally in highlight environments (Warren et al. 2000). Because a large carboxylation capacity potentially uses all available reducing power in the leaves of eucalypts, it is likely to leave limited reducing power for the MEP pathway under stress-free conditions. These characteristics, along with perennial monoterpene storing, may explain why evergreen eucalypts emit less isoprene than deciduous poplars or oaks, which go through annual cycles of senescence and periodic drought. A strong positive correlation between photosynthetic electron transport and isoprene emission is known (Niinemets et

al. 2002). Based on our published results and some indicators ccautiously drawn from the unpublished leaf and canopy gas-exchange data from many eucalypts (Chapter 2, Appendix III), we project that plants with low photosynthetic rates should be high isoprene emitters if they express high electron transport rates relative to carbon assimilation rates (also see **Appendix VII; Dani et al. 2015a**). Elevated CO_2 levels for long periods could cause a significant decrease in maximum carboxylation capacity in many C_3 plants (due to less Rubisco; Norby et al. 1999), although the response of electron transport rate is less obvious (Medlyn et al. 1999). Terrestrial net primary productivity (NPP; ~50 PgC/yr) is sensitive to changes in global temperatures and could decline in future due to unprecedented warm temperatures and extended periods of drought (Melillo et al. 1993; Zhao and Running, 2010). We propose that improving global emission algorithms requires a broad consensus of species' responses to drought. Obtaining a reliable global atlas of tolerances for abiotic stresses among major plant biomes is crucial to understanding the future of plant isoprene emissions in a high-CO_2 world.

Drought-induced increase in concentration of soluble sugars in the cytoplasm is shown to have no bearing on isoprene emission and this raises questions about the import of cytosolic sugars into chloroplasts under severe drought (Rodríguez-Calcerrada et al. 2013). A contrarian view would be that static pool of sugars in the cytoplasm is not important in regulating carbon supply to the MEP pathway rather it is the rate of transport of carbon *to and fro* chloroplasts that is key to variations in isoprenoid emission under stress. There are membrane transporters involved in moving phospho-enol-pyruvate (Fischer et al. 1997) and intermediate substrates of the MEP pathway (e.g. IPP) across the chloroplast membrane (Flügge and Gao, 2005). Nevertheless, it is still reasonable to believe that isoprene emission behaviour is predominantly under the influence of pathways occurring within

chloroplast (and not cytoplasm), which compete with the MEP pathway for both photosynthetic carbon and reducing power (also see Appendix VII).

The questions surrounding photorespiration are relevant also to the data presented in **Chapter 4**, where we show preliminary evidence for the inconsistency in the phenomenon of *post-illumination* monoterpene burst (*PiMB*) in *Quercus ilex* and *Eucalyptus camaldulensis*. The results show that *PiMB* in *Q.ilex* is potentially different from *post-illumination* isoprene burst (in poplars) and more work is underway in our labs in Australia and Italy, to understand the mechanisms underneath these differences. One of the more intriguing observations in Chapter 4 is the contrasting post-illumination behavior of isoprene and monoterpenes in *Eucalyptus camaldulensis*. The absence of a clear *post-illumination isoprene burst* (the first peak) and the selective emission of monoterpenes in the dark in *E. camaldulensis* suggests substrate limitations on isoprene synthesis. However, the secondary smaller burst in isoprene in the dark is intact in *E. camaldulensis*. It was shown recently that direct (substrates) and indirect (re-assimilated) carbon from photorespiration could be channeled towards MEP pathway and isoprene synthesis especially under low CO_2 and high temperature stress scenrios (Jardine et al. 2014). We need know whether a primary burst during *PiMB* is affected by potential re-fixation of photorespiratory CO_2 that could compete with *PiMB* for ATPs and NADPHs in the dark. Given the unequivocal positive effect of temperature on instantaneous and post-illumination behaviour of isoprene emission (Li et al. 2011), new experiments are needed for a comprehensive analysis of *post-illumination monoterpene burst* (*PiMB*) at different temperatures, which will help quantify the relationship between photorespiration and its influence on isoprenoid emission rates in trees from different habitats.

The effect of high temperature in increasing isoprenoid emission is well researched both physiologically and more recently biomechanistically (e.g. Velikova et al. 2011; Pollastri et al. 2014). However, the role of light (intensity, quality and duration) in either accentuating or attenuating the effects of temperature is a complicated problem empirically. While we are still a long way away from mechanistically incorporating clock/time signal transduction in plant emission models, yet it is becoming clear that circadian controls on isoprene emission needs to be an important parameter in refining global isoprene emission models (Hewitt et al. 2011). In **Chapter 5**, we present new experimental leads to show the importance of separating the effects of temperature and photoperiod on circannual (seasonal) cycles in volatile emissions. **We show that diurnal variation in isoprene emission in *Eucalyptus globulus* is entrained by temperature and is photoperiod-gated (Chapter 5). Photoperiod-dependent temperature entrainment of seasonal emissions has significant implications for future projections in global emission because anthropogenic greenhouse gas emissions is increasing global temperature but photoperiod being an external (fixed) variable, remains unaffected by temperature-CO_2 feedback dynamics of the Earth.** The results emphasize the need to separate photoperiod and temperature effects on seasonal emissions, which could help refine the regional discrepancies in emission projections given that inter-specific variation in emission rates among tree genera has a complex annual phenology component built into it. A key step would be to incorporate an annual photoperiod function dependent on the Cumulative Radiative Force (e.g. in $W/m^2/day$) in emission models. This would be akin to Growing Degree Days (GDD), a parameter conventionally used to incorporate phenology in various dynamic global vegetation models (Foley et al. 1996; Krinner et al. 2005).

In **Chapter 6** (published), we provide a novel hypothesis attempting to explain the origin and evolution of isoprene emission capacity in land plants (**Dani et al 2014b**). The

question of why only some plants emit isoprene has bothered many isoprenoid researchers for decades and have led to numerous opinions, arguments and hypotheses (cited in Chapter 6). Yet, a definitive answer has eluded all and the mystery surrounding the origin and occurrence of isoprene emission remains. **Combining a comprehensive analysis of emission traits across the whole plant kingdom (including bryophytes, see Appendix VI) and original insights into the evolutionary physiology and genetics of fast growing trees, we provide a novel and conclusive argument that the occurrence of isoprene emission in land plants is associated with the genera that are fast growing, long-lived and species-rich (Chapter 6).** Although the chapter focusses on isoprene emission in plants, the views presented in the article could have deeper implications on the way we understand and analyse plant evolution. **Chapter 7** (published) **identifies the key tenets of the hypothesis presented in Chapter 6 and showed how fundamentally similar selection criteria operate at different levels of complex organization of life and lead to similar evolutionary trajectories (Dani et al. 2015b).** The discussion reviews a broad range of evidence to show how simple chemical derivation (reduction), highly reactive hydrocarbon chemistry, a wide range of ecological benefits (strong selection) and an unlimited scope for diverse structural configurations have contributed to repeated emergence and functional diversification of biogenic volatile isoprenoid emission in evolutionarily distant and unrelated living systems. The chapter is careful in ***not* attributing** 'motifs' to the process of natural selection, and is conscious of the inappropriate tendency in many scientific disciplines to analyse and describe evolutionary changes as 'desirable' changes, which is an anathema to the theory of natural selection.

We are still aiming at discovering metabolic and biophysical aspects of isoprenoid emissions in many non-model organisms, and such information is likely needed to further decode the complexity of volatile emissions in higher plants. Eucalypts, as it is evident

from this thesis, offer myriad opportunities to investigate isoprenoid emission as a model biochemical process and ecophysiological trait. Our understanding of monoterpene biosynthesis (using *PiMB* as a model), monoterpene storage and emission in eucalypts has important ecological (e.g. antiherbivory, forest fires) and economic (terpene based fuels) facets. Now that a high-resolution whole genome sequence of *Eucalyptus grandis* is available (Myburg et al. 2014), the next logical step would be to decode the complex relationships between phenotypic diversity and emission capacities of eucalypts. There are many hundreds of *Eucalyptus* species and sub-species with both broad and narrow bio-geographical ranges and there is no better system than eucalypts to answer questions pertaining phenotypic plasticity and its influence on ecophysiology of isoprenoid emission. The eucalyptus isoprenoid model has immense potential to not only nurture breakthroughs in scientific research, which is our primary concern, but also sustain research activities by many socioeconomic benefits through industrial applications, a necessary aspect of scientific research in 21st century. Despite significant scientific progress, plant isoprenoid emission retains plenty of mystery to kindle the imagination of generations of researchers to come.

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Appendices[9]

[9] Appendix I : New Phytologist (Review)
Appendix II : Illustrative GCMS Analysis
Appendix III : Supplementary material for Chapter 2
Appendix IV : Supplementary material for Chapter 3
Appendix V : Supplementary material for Chapter 5
Appendix VI : Supplementary material for Chapter 6
Appendix VII : Plant Signaling & Behavior (Addendum)

Appendix I

New Phytologist

Review

Research review

Volatile isoprenoid emissions from plastid to planet

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Key words: biochemical trade-offs, biogenic volatile organic compounds (BVOCs), CO_2 response, drought response, ecological strategies, leaf economic traits, mechanistic model, vegetation emissions.

Summary

Approximately 1–2% of net primary production by land plants is re-emitted to the atmosphere as isoprene and monoterpenes. These emissions play major roles in atmospheric chemistry and air pollution–climate interactions. Phenomenological models have been developed to predict their emission rates, but limited understanding of the function and regulation of these emissions has led to large uncertainties in model projections of air quality and greenhouse gas concentrations. We synthesize recent advances in diverse fields, from cell physiology to atmospheric remote sensing, and use this information to propose a simple conceptual model of volatile isoprenoid emission based on regulation of metabolism in the chloroplast. This may provide a robust foundation for scaling up emissions from the cellular to the global scale.

Introduction

Land vegetation is the principal non-industrial source of biogenic volatile organic compounds (BVOCs) released to the global atmosphere (Denman *et al.*, 2007). Of a total BVOC emission of *c.* 1 Pg C a^{-1}, isoprene and monoterpenes emitted by leaves represent by far the largest fraction (Arneth *et al.*, 2008). These reactive compounds play a fundamental role in determining the atmospheric content of greenhouse gases and pollutants, especially tropospheric ozone, methane, and secondary organic aerosol (Arneth *et al.*, 2010; Carslaw *et al.*, 2010). Because high temperatures stimulate emissions, future projections of changes in atmospheric composition and air quality depend on how these emissions will change.

Modelling of BVOC emissions has generally centred on the quantification of leaf emission capacities (E_c), the emission rates obtained under standard light and temperature conditions (Guenther *et al.*, 1995). Temporal and spatial variation of emissions has been derived by modifying E_c using empirical equations describing observed short-term controls by temperature and light, and long-term controls by antecedent weather conditions and environmental and biotic stresses (e.g. Guenther *et al.*, 2006). Emission capacities were initially considered to be species-specific constants. Several lines of evidence now show that E_c acclimates seasonally and over environmental gradients (Niinemets *et al.*, 2010), but global emission models still set fixed values for plant or vegetation functional types that determine maximum emission rates (e.g. Guenther *et al.*, 2006; Arneth *et al.*, 2007a; Heald *et al.*, 2009; Pacifico *et al.*, 2011). The ever-increasing complexity of these models largely reflects attempts to cope empirically with variations in E_c (Monson *et al.*, 2012).

Monson *et al.* (2012) argue that the use of empirical functions to describe the relationships between emission rates and

environmental variables is unsatisfactory, and particularly the use of serial multipliers based on single factor relationships to account for co-variation of environmental variables. They make a strong case for the need to base modelling on a fundamental understanding of plant biology. Here we argue that progress in understanding the biological foundations of isoprenoid emissions is sufficient to propose a simple conceptual model of isoprene emission that, we believe, will allow construction of a process-based model that will not require a proliferation of empirical specifications.

The emerging new understanding of volatile isoprenoid emissions originates in disparate fields including molecular biology, plant physiology, chemical ecology and atmospheric science. By combining evidence on the regulation of isoprene and monoterpene production with current understanding of their function in plants, we can explore the controls of emissions in a more fundamental way. Well-established findings include the ubiquity of the gene encoding the monoterpene synthase enzyme (Tholl *et al.*, 2011), in contrast with isoprene synthase which is found in a more limited number of higher plant clades (Sharkey & Yeh, 2001); the high short-term sensitivity of isoprene emission to temperature (reviewed in Arneth *et al.*, 2010 and Niinemets *et al.*, 2011); and the inhibitory effect of high CO_2 concentrations on isoprene emission (e.g. Calfapietra *et al.*, 2008; Wilkinson *et al.*, 2009; Possell & Hewitt, 2011). Recent discoveries (reviewed in Loreto & Schnitzler, 2010) include confirmation of the long-hypothesized protective function of isoprene emission (against high-temperature stress and reactive oxygen species (ROS)), established through genetic manipulation experiments (Behnke *et al.*, 2007; Velikova *et al.*, 2011), and studies of atmospheric column concentrations of formaldehyde (HCHO) – a major isoprene oxidation product – which show the dominant role of temperature variations in determining BVOC emissions at the ecosystem level. Based on current understanding of the physiological and environmental controls on isoprenoid synthesis, we introduce key elements of a new quantitative modelling approach that, while being parsimonious, is firmly based on experimental plant biology. We consider regulation of emissions at the biochemical level first, then the function and control of emissions at the whole-plant level, and finally the emergent behaviour of emissions in ecosystems as seen 'top-down' from the atmosphere.

Biochemical controls and trade-offs

Isoprene and monoterpenes are synthesized via the plastidic 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Lichtenthaler, 1999), which is also the beginning of the synthesis route for essential metabolites including abscisic acid, photoprotective compounds (carotenoids and tocopherol) and the phytol side-chain of chlorophyll. Demand for the various downstream products of the MEP pathway can be a significant drain on photoassimilates, energy supply and reducing power (Loreto & Sharkey, 1993; Owen & Peñuelas, 2005; Li & Sharkey, 2012). When the production of different metabolites is viewed in terms of the overall allocation of carbon and energy supplies, trade-offs between the attainment of optimal photosynthetic rates, components of the photosynthetic apparatus (e.g. light-harvesting pigments) and secondary metabolites (e.g. volatile isoprenoids) are seen to be inevitable.

The regulatory network components of the MEP and associated pathways are illustrated in Fig. 1(a) (see also Supporting Information Notes S1). In the accompanying conceptual model (Fig. 1b), we distinguish short (seconds to minutes, A), medium (hours to days, B) and long (weeks to years, C) time-scales of regulation. On *short* time-scales, constitutive emission levels are linked to substrate availability. Isoprene emission, in particular, is strongly determined by the pool size of one of its immediate precursors, dimethylallyl pyrophosphate (DMAPP) (Rasulov *et al.*, 2009a). Substrate-induced surges through the MEP pathway under non-steady-state conditions are constrained by key reactions such as the conversion of methylerythritol cyclodiphosphate to hydroxymethylbutenyl diphosphate (Li & Sharkey, 2012). On *medium* time-scales, transcription rates of enzymes come into play (Mayrhofer *et al.*, 2005). Most genes in the MEP pathway have light-regulated circadian elements (Cordoba *et al.*, 2009) along with putative heat-shock promoter elements upstream of their initiator codon (Sharkey *et al.*, 2008). Hence, it is likely that transcriptional regulation of isoprenoid emission occurs during transient periods of heat and oxidative stress. On *long* time-scales, activation of genes from other pathways (e.g. carotenoid biosynthesis) needs to be considered as linked to volatile isoprenoid emission (Owen & Peñuelas, 2005).

The adaptive significance of isoprenoid emissions

Isoprene is thought to fulfil a protective role in isoprene emitters, either by quenching ROS that are produced in response to high temperatures and excessive light intensities as well as to externally imposed oxidative stress (e.g. high ozone concentration) (Vickers *et al.*, 2009a; Jardine *et al.*, 2011) or by stabilizing thylakoid membranes (Velikova *et al.*, 2011). Recent experimental evidence suggests that stabilization of thylakoid membranes by isoprene reduces the formation of ROS (Velikova *et al.*, 2012). Monoterpenes are known to have a wider variety of functions, including signalling and deterrence of herbivory (Dicke & Baldwin, 2010). Some species store monoterpenes in specialized organs, releasing them by evaporation in response to warming or after mechanical stresses. But many other species emit monoterpenes constitutively in a similar light- and temperature-dependent way to isoprene. It has been proposed that the emission of monoterpenes in these species has a similar function to that of isoprene. Monoterpene emission is elicited by stresses (Loreto *et al.*, 2004). Even in normally non-emitting species, emissions of monoterpenes can be induced by stress (Niinemets *et al.*, 2010), and stress may induce monoterpene emissions instead of isoprene emission (Brilli *et al.*, 2009).

Thus, an emerging view is that volatile isoprenoids in general are important agents in cellular protection from ROS generated during stress events that impair optimal coupling of light and dark reactions within the chloroplast (Loreto & Schnitzler, 2010; Velikova *et al.*, 2012). Although a wide range of strategies to cope

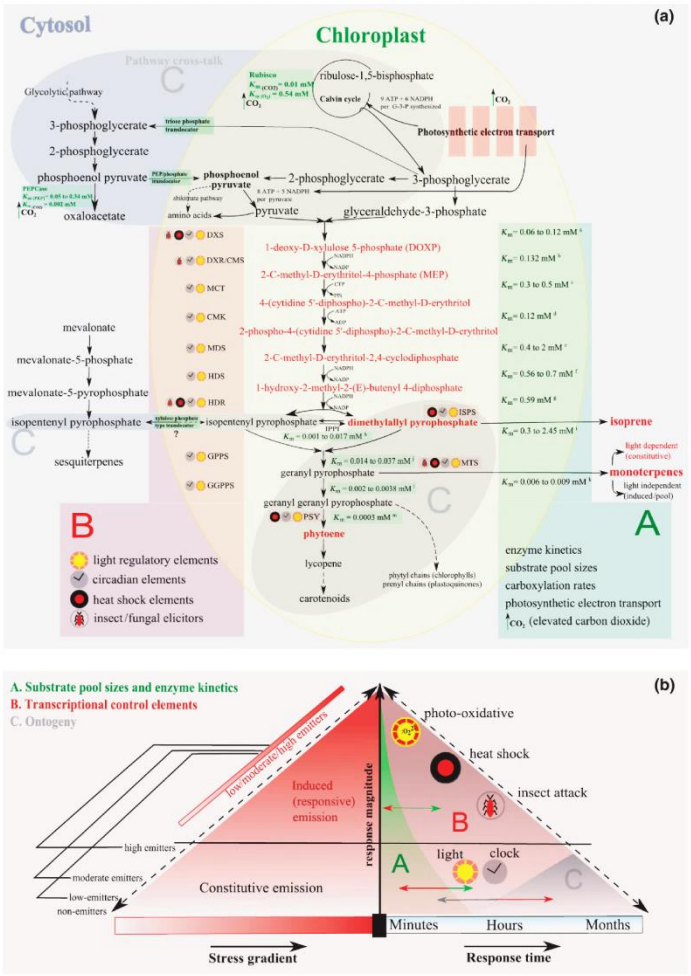

Fig. 1 (a) Summary of the biosynthetic pathways closely linked to plant isoprenoid emission via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. (b) Conceptual representation of diversity in constitutive and inducible emission capacities amongst plant genera juxtaposed with biochemical regulatory elements and their operating time-scales. In (a) and (b), zone A corresponds to constitutive emission controlled by substrate pool limitation; B denotes the transcriptional controls on transient maximum emission; C identifies significant pathway trade-offs influencing, for example, seasonal variability. (See Supporting Information Notes S1 and S5 for references.)

with oxidative stress has evolved in plants, the induction of volatile isoprenoids is important because it can be activated rapidly.

The idea of functional 'substitutability' between isoprene and monoterpenes is consistent with the marked L-shape pattern shown in Fig. 2, which illustrates that species with moderate to high volatile isoprenoid emissions predominantly (or entirely) emit either isoprene or monoterpenes at any given time (the data in Fig. 2 are from studies in which both were measured; see Notes S2). The L-shape pattern is largely driven by species that do not store monoterpene. In species that emit both isoprene and monoterpene simultaneously, the trade-off is manifested as an inverse relationship between the two emission capacities (inset, Fig. 2). This inverse relationship may reflect a competition between monoterpene and isoprene biosynthesis for common precursors and reducing power.

Plants that do have isoprene synthase preferentially emit more isoprene than monoterpenes. This makes the lower affinity of isoprene synthase for its substrate DMAPP (Michaelis–Menten coefficient (K_m) of 0.5–2.5 mM; see Notes S1) compared with that of geranyl pyrophosphate synthase (GPS) for isopentenyl pyrophosphate (IPP) and DMAPP (< 0.05 mM) somewhat puzzling. Moreover, the affinity of monoterpene synthase for geranyl pyrophosphate (GPP) synthesized by GPS is even higher (K_m of 0.006 mM). The substrate pool size of GPP is estimated to be much larger than that of DMAPP, suggesting that the kinetics of GPP synthase strongly influences GPP availability for

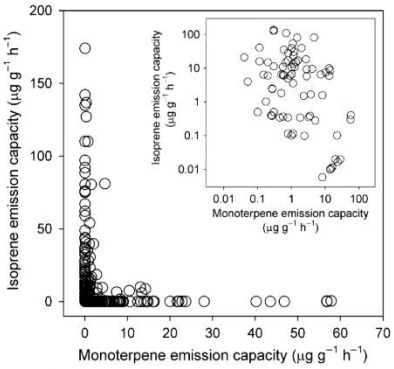

Fig. 2 Trade-off between isoprene and monoterpene emission capacities. The main plot, the 'L-graph', is based on 403 data points representing 192 species from 48 plant families, drawn from 35 individual studies on field-sampled adult plants. Non-zero emissions for *both* isoprene and monoterpene were reported for 80 of 403 cases; these are shown again in the inset panel, on $\log_{10}$-scaled axes.

downstream processes. It is unclear how adequate substrate is still available for isoprene synthesis, given the bias towards synthesis of IPP over DMAPP, in emitting species. It is possible that the DMAPP pool co-limits isoprene and GPP synthesis, depending on the diurnal and seasonal variations in the relative V_{max} (maximum enzyme reaction rate) values for the two enzymes (Fig. 1).

Isoprenoid emissions and plant ecological strategies

There are broadly predictable ecological trends in the predominance of isoprene vs monoterpene emissions (Fig. 3). The strongest association is with species' shade tolerance, which is an indicator of successional status. Early successional and light-demanding plants have a greater tendency to produce isoprene (Fig. 3a). This reflects the fact that coping with oxidative stress and/or high temperature is likely to be a greater challenge in higher irradiance or higher-light sites, and that there are few 'excess' electrons (and carbon chains) for isoprene production in low light. Isoprene emission is also associated with leaf traits characteristic of species with rapid growth in high-resource (including high-light) environments, including high photosynthetic capacity (A_{max}) (Fig. 3b), short leaf lifespan (Fig. 3c), and high specific leaf area (SLA) (Fig. 3d). Investment in isoprene, being costly because of its high volatility, can be viewed as a fast-response strategy that pays off in an environment where transient stress is frequent or as a component of a fast-growth strategy in nonlimited resource environments. By contrast, investment into monoterpenes could be seen as more efficient under conditions where constitutive emissions are required over long periods, for example to confer protection in longer-lived leaves against herbivory.

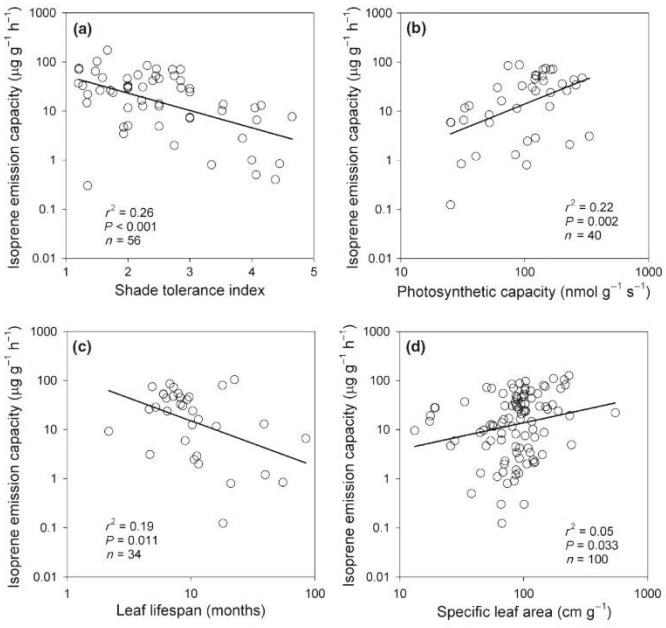

Fig. 3 Relationship between isoprene emission capacity and traits related to species' ecological strategies: (a) shade tolerance index, (b) photosynthetic capacity, (c) leaf lifespan, and (d) specific leaf area (SLA). Data are for 134 tree and shrub species from a broad range of families and vegetation types; in each graph each data point represents a different species. SLA data were sourced from the same publications as the emissions data; shade tolerance indices are from Niinemets & Valladares (2006); photosynthesis and leaf lifespan data were derived from a variety of published and unpublished sources (see Supporting Information Notes S2 and S5). Photosynthetic capacity refers to measurements made in the field under near-optimal light, temperature and soil moisture conditions.

Decoupling of isoprenoid emissions and photosynthesis

Although isoprenoid emissions are dependent on photosynthesis for the supply of energy (ATP), reducing power (NADPH) and carbon skeletons, several environmental and ontogenetic factors decouple the two processes. For instance, both high temperatures and soil water deficits reduce photosynthesis, while isoprene emission can continue at a high level (reviewed in Niinemets, 2010). Isoprene emissions from leaves grown at above-ambient CO_2 concentration are inhibited, while emissions from leaves grown at subambient CO_2 concentration are increased (see next section). Light-dependent isoprene emission has been observed from leaves that have been severed from the stem and ceased to photosynthesize (Loreto & Schnitzler, 2010; Brilli *et al.*, 2011). Finally, a lag between photosynthesis and isoprene emission in developing leaves has been reported in several temperate and tropical plants (see Table S1).

The lag between photosynthetic competency and measurable isoprene emission is not simply a function of leaf expansion. It varies from a few days to several weeks, depending on the growth temperature (see Notes S3 and Table S2) and appears to be under transcriptional control, that is, there is delayed expression of the gene encoding isoprene synthase (Wiberley *et al.*, 2005; Sharkey *et al.*, 2008). The adaptive significance of this genetically programmed delay is uncertain. We suggest that it is linked to the priority for synthesis of more essential isoprenoids during early leaf development. The picture is less consistent in ageing leaves: some studies suggest that the biochemical capacity to produce isoprene is unaffected by senescence, some that isoprene declines before photosynthesis, and some that measurable isoprene emission persists in senescing leaves even after the cessation of photosynthesis (Table S2). In poplars (*Populus* × *euroamericana*) grown at elevated CO_2, isoprene emission was sustained for longer periods in senescing leaves, while the decline in photosynthesis was accelerated (Centritto *et al.*, 2004). Under these conditions, Tallis *et al.* (2010) showed increased expression of genes involved in glycolysis, suggesting that PEP (phosphoenolpyruvate) from glycolysis, translocated to the chloroplast, may provide the substrate for sustained isoprenoid emission in senescing leaves (Fig. 1; Loreto *et al.*, 2007).

The delayed onset of volatile isoprenoid emissions is reflected in large-scale observations over vegetation with a strong component of seasonally deciduous trees (see Notes S4). Remotely sensed HCHO concentration can be used as a proxy for regional volatile isoprenoid emission (e.g. Barkley *et al.*, 2009). In temperate latitudes (Fig. 4), HCHO lags the increase of remotely sensed leaf area index (LAI) in spring. There is no such clear signal in the autumn: the decline in HCHO occurred before the decline in LAI in 2007 and after the decline in LAI in 2006. The lags differed between the 2 years analysed, suggesting a possible route to investigate the environmental cues involved from a 'top-down', ecosystem-level perspective. The interpretation of the HCHO signal in the tropics is complicated by the substantial contribution of pyrogenic emissions (Gonzi *et al.*, 2011). Nevertheless, disregarding situations when the peak in HCHO occurs in the dry season when the trees are leafless, tropical deciduous forests and savannas show a quite different pattern from temperate deciduous forests (Fig. 4). Low isoprenoid emissions occur in the wet season when air and canopy temperatures are at a minimum.

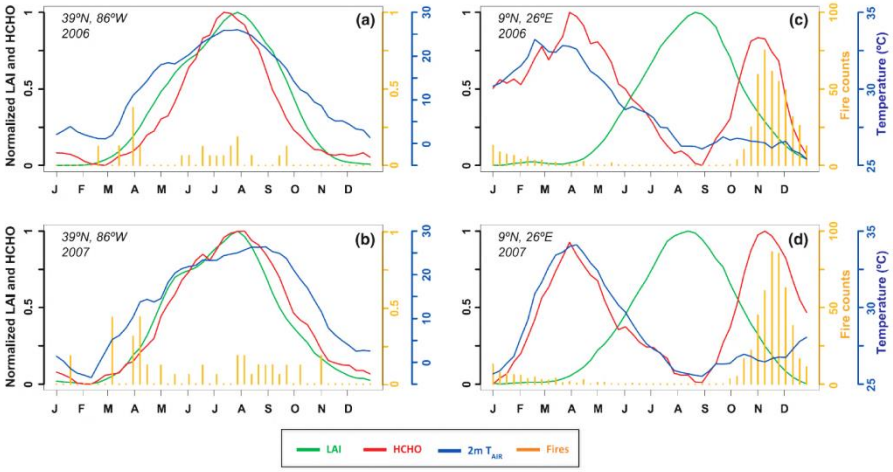

Fig. 4 The relationship between normalized leaf area index (LAI), 2-m air temperature in degrees Celsius (2m T_{AIR}) and volatile isoprenoid emissions indexed by the normalized remotely sensed atmospheric column concentration of formaldehyde (HCHO). Data are from a temperate (a, b) and tropical (c, d) broad-leaved deciduous forest during 2006 and 2007. The second HCHO peak in the tropical forest (c, d) is attributed to biomass-burning emissions, as shown by the remotely sensed fire counts (FIRE) (see Supporting Information Notes S4).

Thus, the seasonal cycles of LAI and HCHO are not correlated in the tropics, and HCHO concentration tracks temperature rather than LAI.

Isoprene responses to CO_2 and drought

The experimentally observed responses of isoprene emissions to CO_2 and water stress are fundamental in the context of global climate change and need to be accounted for in models (Arneth *et al.*, 2007b; Heald *et al.*, 2009; Young *et al.*, 2009; Pacifico *et al.*, 2012). The effect of high CO_2 concentration in suppressing isoprene emission is sufficient to strongly reduce or even offset the increase in isoprene emission resulting from high temperatures, which in turn significantly affects ozone projections (Young *et al.*, 2009; Pacifico *et al.*, 2012).

The effect of CO_2 concentration on isoprene production is in the opposite sense to the CO_2 effect on photosynthesis. Plants grown at high atmospheric CO_2 concentrations emit less isoprene than those grown at lower concentrations (Sharkey *et al.*, 1991; Centritto *et al.*, 2004; Scholefield *et al.*, 2004). It has been shown that isoprene emission responds to the leaf-internal CO_2 concentration (c_i), with lower emission rates associated with higher c_i (Monson *et al.*, 2007; Wilkinson *et al.*, 2009; Guidolotti *et al.*, 2011; Possell & Hewitt, 2011). The effect has been found in many plant species, and is persistent – it applies to plants grown in different CO_2 concentrations, as well as in short-term experiments that manipulate ambient CO_2 concentration in order to alter c_i, and to genotypes with different c_i grown under the same CO_2 concentration. Variation of the pool size of DMAPP is the most likely explanation for the CO_2 dependence of isoprene synthesis as seen in short-term experiments (Rasulov *et al.*, 2009b). Although not explicitly identified, this regulation could occur because of changes in DOXP (1-deoxy-D-xylulose-5-phosphate)/MEP pathway enzymes upstream of isoprene synthase, changes in the energy status of chloroplasts, or competition for DMADP between other key metabolic pathways (Sun *et al.*, 2012). When plants are grown under different CO_2 concentrations, it is likely that transcriptional regulation of isoprene synthase activity is also involved (Scholefield *et al.*, 2004; Possell & Hewitt, 2011).

We propose a unifying mechanistic hypothesis, based on the requirement of energy and reducing power for isoprene biosynthesis. NADPH is needed in order to produce DMAPP (Rasulov *et al.*, 2011). When photosynthesis is electron transport-limited (at high c_i and/or low light), the shortfall of ATP and NADPH for CO_2 assimilation will cause a deficit of reducing power to transform carbohydrates into DMAPP. When photosynthesis is Rubisco-limited (at low c_i and/or high light), the plant will use a part of the excess of ATP and NADPH (resulting from the excess of electrons produced by photochemical reactions) to reduce carbohydrates to DMAPP. This hypothesis predicts that isoprene emission will increase in response not only to excessively high temperatures but also, more generally, to any situation where light availability exceeds photosynthetic capacity. The fraction of the carbon assimilation allocated to isoprene production increases with light intensity, even when photosynthesis is light-saturated (Sharkey & Loreto, 1993), consistent with this hypothesis. An important corollary is that stomatal closure in response to dry conditions will increase isoprene emission by lowering c_i and thereby increasing the supply of electrons relative to the carbon fixation rate. Therefore, this hypothesis simultaneously accounts for observed responses of isoprene emissions to CO_2, and to drought – because under drought conditions c_i is reduced, and photosynthetic carbon fixation is reduced, while electron transport is maintained.

The simple model proposed here is based on the Farquhar & Von Caemmerer (1982) model of leaf photosynthesis, which assumes that photosynthesis is limited by either the electron transport supply or the rate of carbon fixation by Rubisco. The excess or deficit of electrons produced by photochemical reactions during photosynthesis can be calculated as the difference between the total photosynthetic electron flux and the total flux of electrons used for carbon assimilation (Fig. 5a). We argue that the rate of isoprene emission (I; nmol m^{-2} s^{-1}) depends on excess reducing power, which is increased by the electron-transport supply (J; μmol e^- m^{-2} s^{-1}), and reduced by the electron-transport requirement of the dark reactions of photosynthesis, calculated as

$$V_{\mathrm{cmax}} \frac{C_i + 2\Gamma*}{C_i + K_m} \qquad \text{Eqn 1}$$

where V_{cmax} is the maximum rate of Rubisco activity, c_i is the intercellular CO_2 concentration, Γ^* is the CO_2 compensation point in the absence of dark respiration, and K_m is the Michaelis–Menten coefficient.

The isoprene emission rate is thus given by

$$I = a J - b V_{\mathrm{cmax}} \frac{C_i + 2\Gamma*}{C_i + K_m} \qquad \text{Eqn 2}$$

where a and b are empirical parameters: a represents a maximum fraction of the total photosynthetic electron flux used for isoprene synthesis, while b represents the rate at which isoprene synthesis is diminished in proportion to the demand set up by the dark reactions of photosynthesis. Preliminary comparisons with isoprene emissions from two experimental species shows that this modelling approach reproduces observed responses to temperature (Fig. 5b), light intensity (Fig. 5c) and CO_2 (Fig. 5d). This approach is attractively parsimonious, and consistent with the idea that isoprene production is not tightly linked to carbon assimilation. It requires further testing (e.g. for the response to simultaneous perturbations of different factors). Whether it is also applicable to monoterpene emissions, especially in species that emit these compounds in an isoprene-like fashion, also needs to be examined.

Towards greater scientific integration

The processes involved in regulating the emission of volatile isoprenoids operate on many time and space scales, and are consequently studied within several disciplines that do not habitually communicate. Integration across disciplinary boundaries is

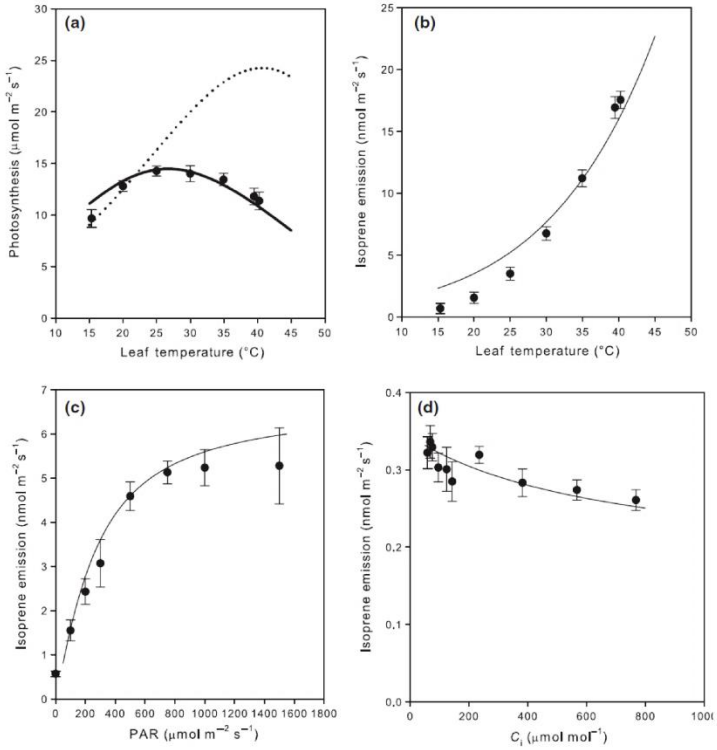

Fig. 5 Characteristic responses of isoprene emission to temperature, incident photosynthetically active radiation (PAR) and intercellular CO_2 concentration captured by a simple model of 'excess' reducing power within the chloroplast. (a) At low temperatures, photosynthesis is limited by electron transport availability (dashed line), whereas at high temperature photosynthesis is limited by Rubisco activity (solid line), resulting in (b) a steep increase in excess reducing power and isoprene emission with temperature (data show measured photosynthesis and isoprene emission in tobacco (*Nicotiana tabacum*) (Vickers *et al.*, 2009a); error bars represent SE). (c) Increase in isoprene emission with PAR can be attributed to the increase in reducing power (data from Vickers *et al.*, 2009b). (d) Decrease in isoprene emission with increasing internal CO_2 concentration can be attributed to an increase in photosynthetic rate which consumes reducing power (data from Possell & Hewitt, 2011).

necessary to develop a more comprehensive understanding of these processes. In particular, better integration of modelling, remote sensing, and experimental plant biology is important to overcome the current paradoxical situation whereby models linking emissions, atmospheric chemistry and climate are strongly data-driven, yet hampered by the lack of data on both the short time-scales needed for derivation of environmental response curves and the longer time-scales needed to characterize the response to environmental change.

The concept of trade-offs at the biochemical level provides a useful framework for explaining and modelling isoprenoid emissions. But more, and more systematic, observations are needed. There are too few measurements of enzyme activities and substrate pools across emitting and non-emitting species to allow cost–benefit analysis for the different 'flavours' of isoprenoid emission. There has been insufficient analysis of the relative costs of alternative stress-protection strategies. Gaps also remain in process understanding at the cellular level. We still do not know, for example, what controls the transport of substrates between the cytosol and the chloroplast.

Plant trait databases that include geo-located records (e.g. Kattge *et al.*, 2011) make it possible to explore the relationships between emissions and other plant characteristics. Such investigations could help to clarify further the interaction between ecological strategies and emissions, or to analyse trade-offs at the species level – for example, what strategies to deal with oxidative stress are used by non-emitters – and to discern macro-scale relationships between emissions and environment.

In response to the need to develop a more biologically robust way of simulating plant emissions (Monson *et al.*, 2012), we have proposed a simple concept for modelling the emission of volatile isoprenoids that is consistent with current understanding of physiological mechanisms, including dependence on reducing power (Li & Sharkey, 2012), and with a diverse set of observations. Although some earlier models have simulated isoprenoid emissions based on assigning a fraction of the total electron transport to the process (e.g. Niinemets *et al.*, 1999; Arneth *et al.*, 2007a), they required empirical modifications to represent CO_2 and drought effects (Monson *et al.*, 2012). While our model appears to capture the expected responses to temperature, light and CO_2, it remains to be shown whether it can reproduce features such as the large diurnal range in emissions, seasonality induced by temperature and water stress variations, short- and long-term responses to CO_2 concentration, and global patterns emerging from satellite observations of HCHO and other BVOC oxidation products. Nevertheless, application and extension of this framework should provide more robust estimates of isoprenoid emission in a changing environment.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Information on K_m values

Table S2 Observational or experimental evidence for lag in emissions

Notes S1 Source of K_m data.

Notes S2 Trait and isoprene database.

Notes S3 Experimental and field evidence for seasonal leads and lags between isoprene emission and photosynthesis.

Notes S4 Analysis of isoprene emissions at a regional scale.

Notes S5 References.

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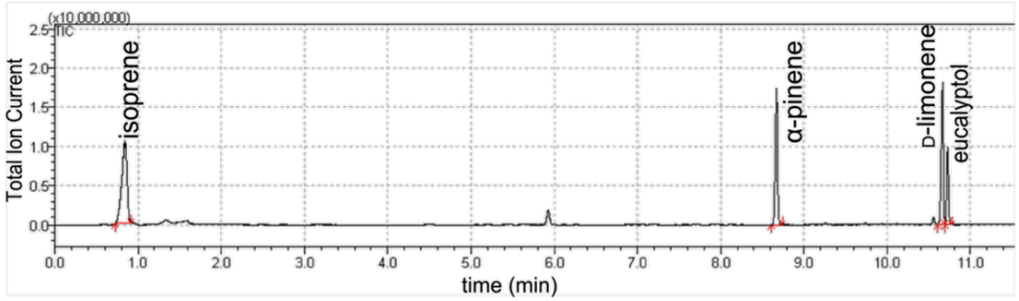
Appendix II

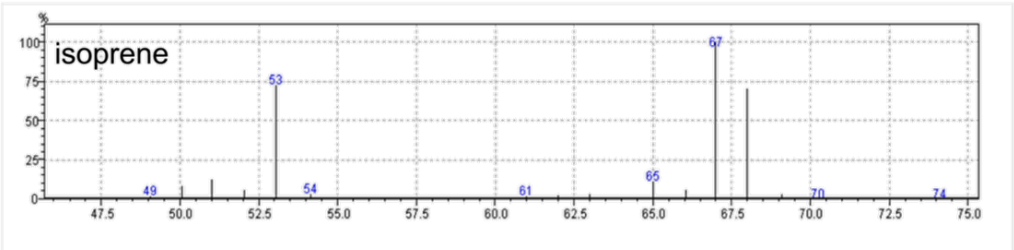
Illustrative Gas Chromatographic and Mass Spectral Analysis

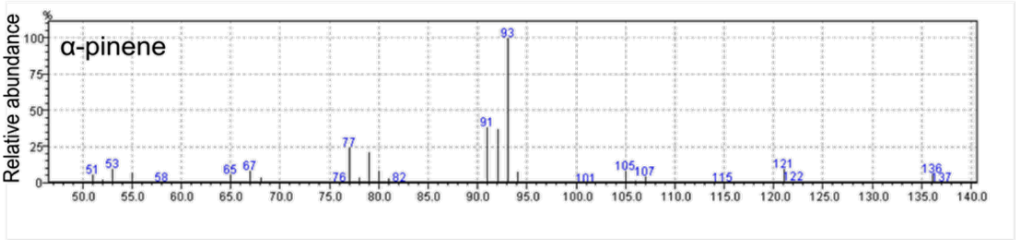
Volatile hydrocarbon	IUPAC name	Molecular formula	Molecular weight	Retention time (min)	Major ion fragments
Isoprene	2-methyl-1,3-butadiene	C_5H_8	68.12	0.837	67, 53
α-pinene	2,6,6-trimethyl bicyclo hept-2-ene	$C_{10}H_{16}$	136.23	8.670	93
D-limonene	1-Methyl-4-(1-methylethenyl)-cyclohexene	$C_{10}H_{16}$	136.23	10.662	68, 67, 93
eucalyptol	2,2,4-trimethyl-3-oxabicyclo octane	$C_{10}H_{18}O$	154	10.728	81

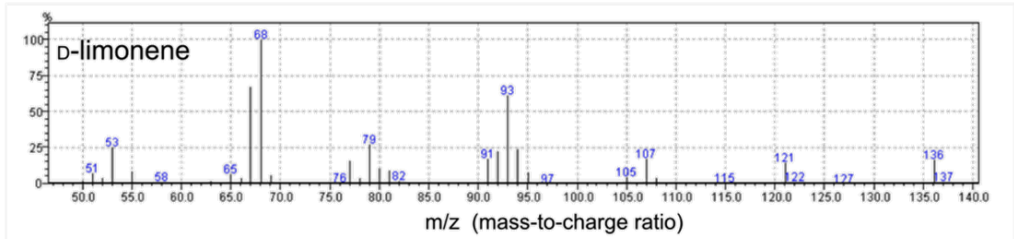
Example GC MS chromatogram of a standard run



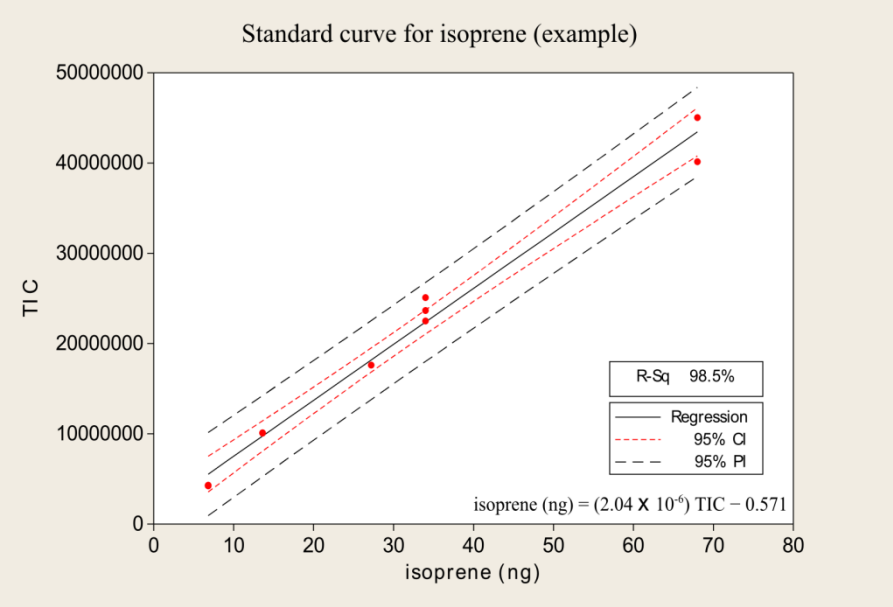
Relative abundance of ionic fragments of volatile hydrocarbons (example mass spectra)

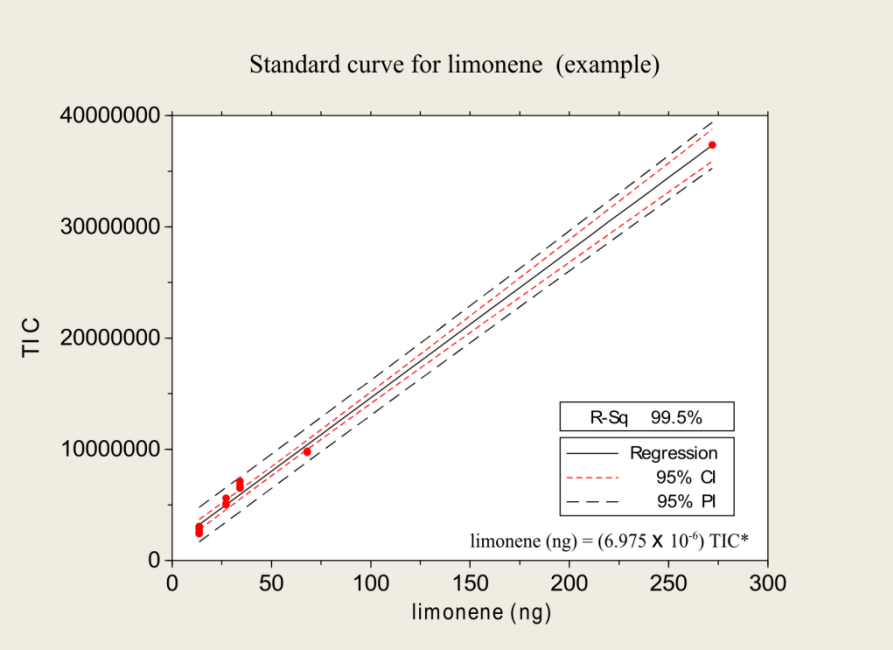




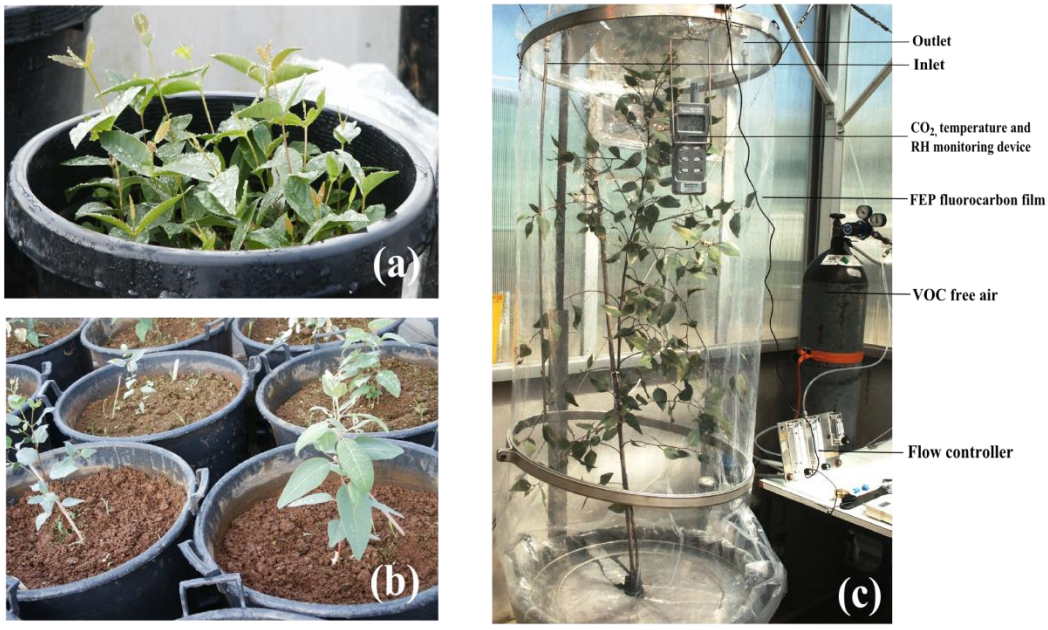


Example standard curves for isoprene and limonene

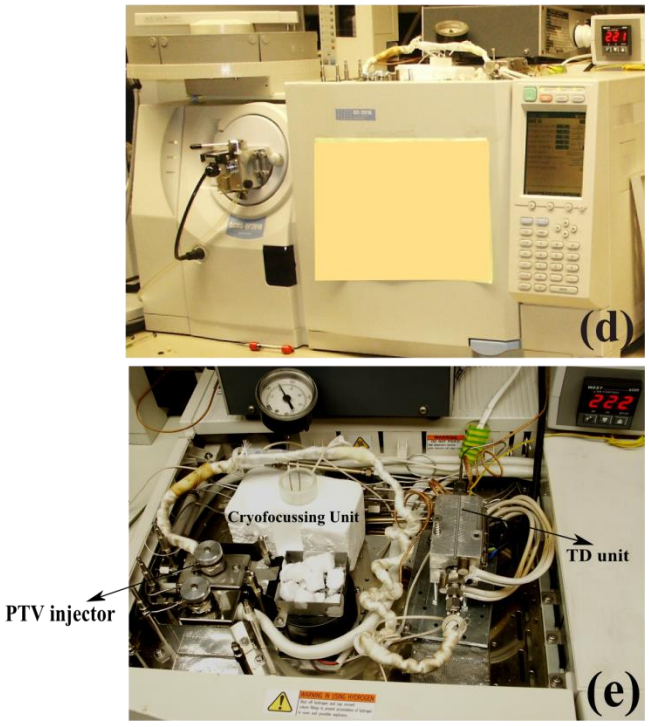




Selected photographs of the experimental methods



(a) Young eucalypt seedlings; (b) Plants growing in 80 kg pots in the glass house ;
(c) Isoprenoid (whole-plant) sampling unit in action (used in initial screening , see Chapter 2) ;
(d) Shimadzu GCMS-QP2010 ; (e) Thermal desorption and cryofocussing unit (later replaced by a TD autosampler)



Appendix III

Chapter 2: Supplementary material

Photosynthesis and isoprene emission in eucalypts from contrasting environments

Figure S1: Geographic distribution (only indicative) of eucalypts chosen for this study.
Map adapted from Wikipedia

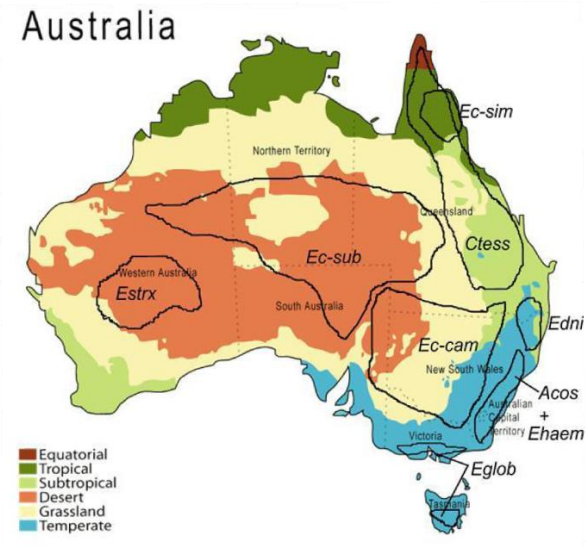


Table S1: Inorganic chemical fertilizer proportions used for growing eucalypts			
Macronutrients	g kg^{-1} of dry soil	gram per 80 kg of soil (per pot)	Purpose
$CaCO_3$	7	560	Reduces soil acidity and provides Ca^{2+}
$MgCO_3$	1.75	140	Reduces soil acidity (mimics dolomite lime) provides Mg^{2+}
$(NH_4)_2SO_4$	2.83	226.4	Nitrogen source
$CaHPO_4.2H_2O$	4	320	Source of Phosphorous
K_2CO_3	2.5	200	Potash (improves phosphate absorption)
$CaSO_4$	1	80	Gypsum improves soil texture
$MgSO_4$	1	80	Provides Mg^{2+}
Micronutrients	mg kg^{-1} of dry soil	mg per 80 kg of soil (per pot)	Purpose
$ZnSO_4.7H_2O$	100	8000	Provide essential metal ions
$CuSO_4.5H_2O$	19.6	1568	
$Na_2MoO_4.2H_2O$	0.03	2.4	
H_3BO_3	0.55	44	
EDTA-Fe III Na salt	4.4	352	

Chapter 2: Supplementary material

Photosynthesis and isoprene emission in eucalypts from contrasting environments

Table S2: Tree seeds obtained from ATSC (CSIRO) at Canberra and FPC at Western Australia.
Species were further short-listed from this group based on soil compatibility and growth response under glass house conditions (Table S3)

Seeds	Seed lot	Location	Lat	Lon	Altitude	Number of parents	Reported viability per 10g
Drought tolerant species							
Eucalyptus striaticalyx	15779	CUE	2653S	11816E	250	14	1650
E. occidentalis	20694	SPA BUNDALEER	3319S	13833E	200	10	1050
E. camaldulensis subsp. *subcinerea*	20713	ARTHUR CREEK	2240S	13638E	270	10	6800
Species from the tropical and the subtropical latitudes							
E. tetrodonta	20584	KATHERINE	1431S	13212E	95	9	232
E. camaldulensis subsp. *obtusa*	13928	VICTORIA RIVER	1535S	13102E	35	10	2600
E. camaldulensis subsp. *simulata*	20651	PALMER RIVER	1606S	14446E	110	10	3000
Corymbia tessellaris	20653	KENNEDY RIVER	1523S	14410E	100	15	2000
Temperate species							
E. dunnii	20979	SPA Hermistons	3519S	14519E	100	12	2400
E. regnans	15160	MT USEFUL	3741S	14632E	950	10	858
E. globulus	17609	WILSONS PROMONTORY	3908S	14625E	60	8	392
E. sideroxylon	15199	43K S WAGGA WAGGA	3524S	14721E	400	10	1450
E. camaldulensis subsp. *camaldulensis*	20437	BARMAH SF	3550S	14507E	100	10	10000
Corymbia maculata	21069	SSO Bunyip	3803S	14542E	100	12	1314

Chapter 2: Supplementary material

Photosynthesis and isoprene emission in eucalypts from contrasting environments

Table S3: Photosynthesis data for eucalypts from LiCor 6400XT

Code on Figure 2S1	Eucalypt	Type of Foliage	Number of replicates		Net assimilation rate at 30 °C (at c_a=400ppm) µmol $m^{-2}s^{-1}$		c_i at c_a=400ppm		stomatal conductance (mol m^{-2} s^{-1})	
			No of plants	No of leaves	mean	std dev	mean	std dev	mean	std dev
Eglob	*Eucalyptus globulus*	Juvenile	5	25	10.9	1.86	177	20	0.09	0.04
Ehaem	*E. haemastoma*	Juvenile	4	08	9.2	3.39	181	20	0.11	0.05
Ctess	*Corymbia tessellaris*	Juvenile	4	28	5.0	1.40	231	12	0.06	0.02
Estrx	*E. striaticalyx*	Adult	4	10	20.2	1.55	252	8	0.31	0.02
Edni	*E. dunnii*	Adult	3	22	8.7	1.52	233	8	0.08	0.02
Ec-cam	*E. camaldulensis camaldulensis*	Adult	4	19	22.9	1.82	227	5	0.23	0.03
Ec-sim	*E. camaldulensis simulata*	Adult	3	32	18.5	2.47	197	9	0.12	0.02
Ec-sub	*E. camaldulensis subcinerea*	Adult	3	20	21.8	2.51	200	5	0.19	0.05
Acos	*Angophora costata*	Adult	4	08	11.6	2.42	200	8	0.15	0.02

Morphological characteristics :
E. globulus, C. tessellaris, had distinct juvenile foliage (sessile-elliptic-opposite-decussate); *E. haemastoma,* had juvenile foliage (petiolate-disjunct-ovate/lanceolate); *A. costata* had some juvenile (sessile-opposite) but mostly metamorphosed intermediate/adult (petiolate-opposite) leaves. The rest of the species had adult foliage.

Chapter 2: Supplementary material

Photosynthesis and isoprene emission in eucalypts from contrasting environments

Table S4: Isoprene emission sampling conditions and emission rates for eucalypts

Eucalypt	Type of Foliage	Whole plant canopy sampling replicates	Head-space temperature (˚C)		Head-space humidity (%)		Isoprene emission rate (nmol m^{-2} s^{-1})	
			mean	std dev	mean	std dev	mean	std dev
E. globulus	Juvenile	4	32.8	1.95	26.3	3.42	3.6	0.83
E. haemastoma	Juvenile	7	33.1	2.87	23.4	4.54	0.3	0.12
C. tessellaris	Juvenile	8	33.6	1.79	27.6	2.98	1.5	0.40
E. striaticalyx	Adult	6	33.5	1.50	29.0	4.28	4.1	0.87
E. dunnii	Adult	6	35.1	1.06	38.5	1.85	0.02	0.00
E. camaldulensis camaldulensis	Adult	3	34.6	2.05	25.0	0.81	5.1	0.25
E. camaldulensis simulata	Adult	3	34.0	0.80	59.6	3.10	1.1	0.30
E. camaldulensis subcinerea	Adult	3	32.3	0.47	32.3	2.05	2.3	0.40
A. costata	Adult	8	31.5	2.29	34.0	6.84	0.47	0.20

Table S5: Maximum Carboxylation rate by Rubisco (V_{cmax}), Instantaneous electron transport rate (J) and the ratio between the two estimates for selected eucalypts (replicates represent independent A-c_i curves obtained from three fully expanded, healthy leaves)

Eucalypt	Replicates	V_{cmax}				J				J/V_{cmax}				
	N	rep1	rep2	rep3	Mean	rep1	rep2	rep3	Mean	rep1	rep2	rep3	Mean	std dev
E. globulus	3	98	128	75	100.33	103	149	87	113.00	1.05	1.16	1.16	1.13	0.06
E. haemastoma	1	NA	NA	107	107.00	NA	NA	115	115.00	NA	NA	1.07	1.07	NA
C. tessellaris	3	82	74	65	73.67	108	99	95	100.67	1.32	1.34	1.46	1.37	0.08
E. striaticalyx	3	243	222	194	219.67	200	197	192	196.33	0.82	0.89	0.99	0.90	0.08
E. dunnii	3	126	110	106	114.00	129	117	113	119.67	1.02	1.06	1.07	1.05	0.02
E. camaldulensis simulata	3	107	117	102	108.67	116	117	105	112.67	1.08	1.00	1.03	1.04	0.04
E. camaldulensis camaldulensis	3	301	261	286	282.67	220	219	220	219.67	0.73	0.84	0.77	0.78	0.05
E. camaldulensis subcinerea	3	351	345	294	330.00	315	309	298	307.33	0.90	0.90	1.01	0.94	0.07
A-c_i curves were not obtained for *Angophora costata*														

Appendix IV

Supplementary information for Chapter 3

Research article in *Plant Physiology*

Increased ratio of electron transport to net assimilation rate supports elevated isoprenoid emission rate in eucalypts under drought

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Table S1: Photosynthesis parameters of *Eucalyptus camaldulensis* subsp. *camaldulensis* in response to drought and photorespiratory stress
(Experiment 3) leaf temperature of 25 ˚C and PAR=1200 µmol $m^{-2}s^{-1}$

Treatment group	Net assimilation rate (µmol $m^{-2}s^{-1}$)	Stomatal conductance (mol $m^{-2}s^{-1}$)	Leaf internal CO_2 at C_a=400 µmol mol^{-1}	Transpiration rate (mmol $m^{-2}s^{-1}$)	Leaf temperature (˚C)	Relative Humidity (%)
2% O_2 + 100% FC	27.5 ± 1.14	0.65 ± 0.06	275 ± 16	7.2 ± 0.15	25.0 ± 0.05	56 ± 2
2% O_2 + 50% FC	5.9 ± 0.39	0.10 ± 0.01	267 ± 11	2.0 ± 0.23	25.2 ± 0.09	41 ± 1
20% O_2 + 100% FC	20.4 ± 0.20	0.52 ± 0.03	302 ± 4	7.7 ± 0.31	25.0 ± 0.07	44 ± 2
20% O_2 + 50% FC	6.6 ± 0.59	0.11 ± 0.02	234 ± 7	2.4 ± 0.33	25.1 ± 0.16	35 ± 2
50% O_2 + 100% FC	10.3 ± 0.69	0.45 ± 0.04	366 ± 7	5.7 ± 6.92	24.9 ± 0.11	52 ± 2
50% O_2 + 50% FC	3.8 ± 0.56	0.14 ± 0.04	348 ± 14	2.8 ± 0.63	25.1 ± 0.08	34 ± 3
All values are Mean ± 1 SE (*N*=5)						

Supplementary Figure S1

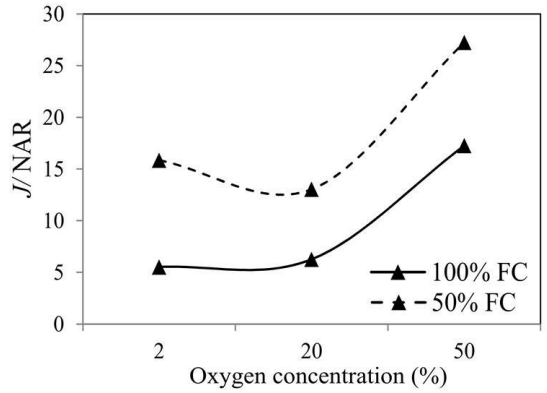

Fig. S1: Electron transport rate to net assimilation rate ratio in response to varying levels of oxygen concentration in *E. camaldulensis* subsp. *camaldulensis* (Experiment 3)

Fig. S1: **Electron transport rate to net assimilation rate ratio in response to varying levels of oxygen concentration in well-watered (100% FC) and droughted (50% FC) *E. camaldulensis* subsp. *camaldulensis*** (Experiment 3)

Supplementary Figure S2

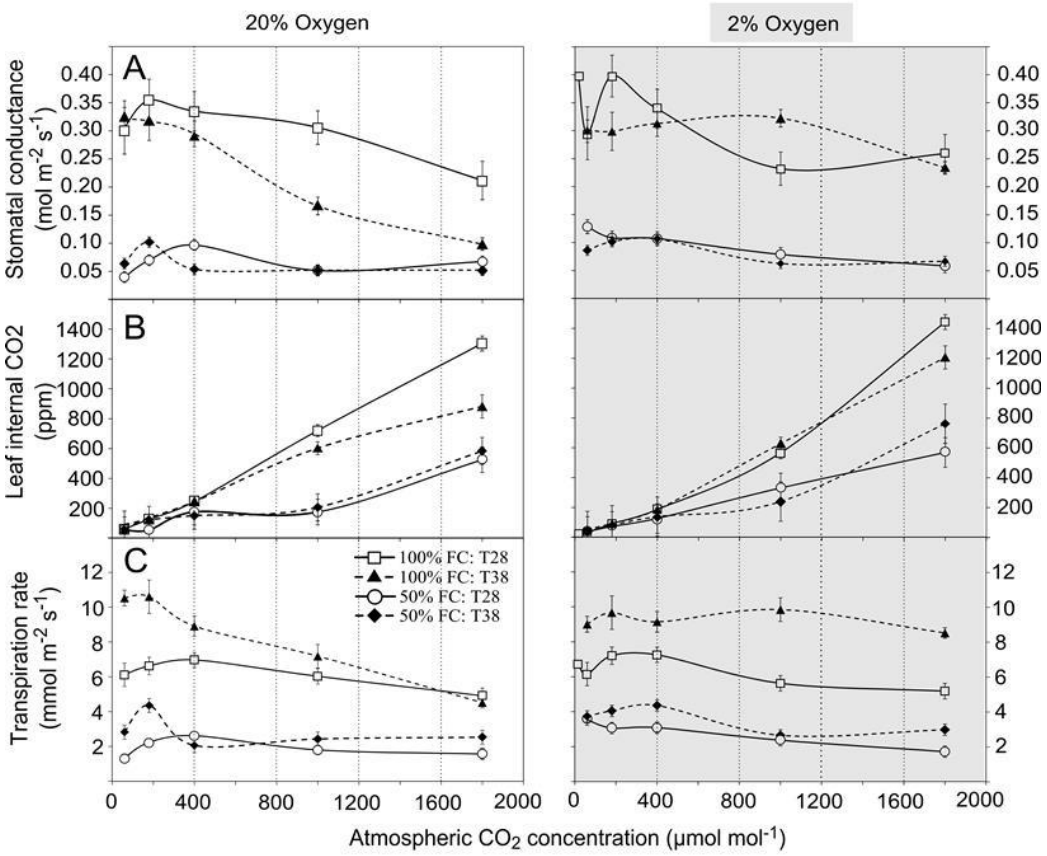

Fig. S2: **Photosynthesis response to short-term heat stress**

Fig. S2: Response of photosynthesis to short-term exposure to heat stress under 20% O_2 (left panel) and 2% O_2 (right panel) over a CO_2 concentration span (60 µmol mol^{-1} to 1800 µmol mol^{-1}) in *Eucalyptus camaldulensis* subsp. *obtusa* acclimated to well-watered condition (100% FC) and drought (50% FC) (A) stomatal conductance (B) leaf internal CO_2 concentration (C) transpiration rate (*N*=6, means ± 1 SE). Note: It needs to be tested whether stomatal limitation on CO_2 diffusion also reduces RuBP regeneration capacity (Tezara et al., 1999) and whether it contributes to increased emission under drought. (Tezara W., Mitchell V.J., Driscoll S.D. & Lawlor D.W. (1999) Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. *Nature* 401, 914-917)

Supplementary Figure S3

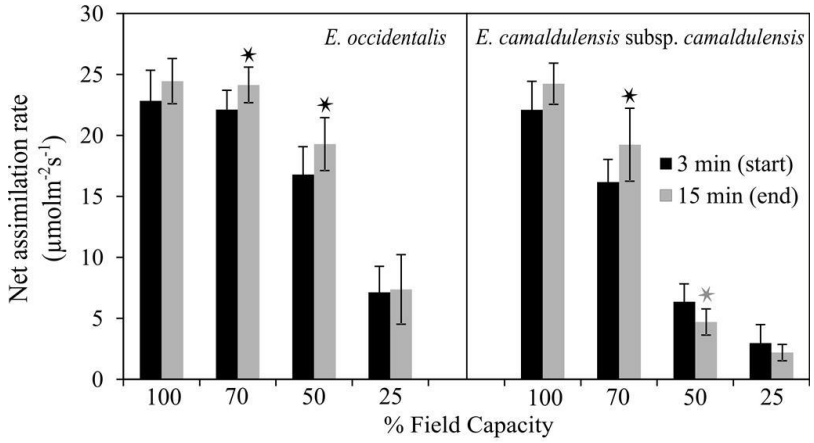

Fig. S3 : Short term (15 min) response of photosynthesis to low O_2 (2% oxygen) (Experiment 1)

Fig. S3: **Short term (15 min) response of photosynthesis to low O_2 (2% oxygen)** at 25 ºC and 400μmol mol^{-1} CO_2 in two eucalypts acclimated to well-watered condition (100% FC) and drought (50% FC) (*N*=4, mean ± SD). In both species net assimilation rate increases and stabilises within 5 minutes (monitored until 15 min) after exposure to low O_2 under well-watered conditions (Black**P*≈0.06);. Photosynthesis declines under low O_2 in *E. camaldulensis* subsp. *camaldulensis* experiencing severe drought (≤50% FC; Grey**P*=0.11)

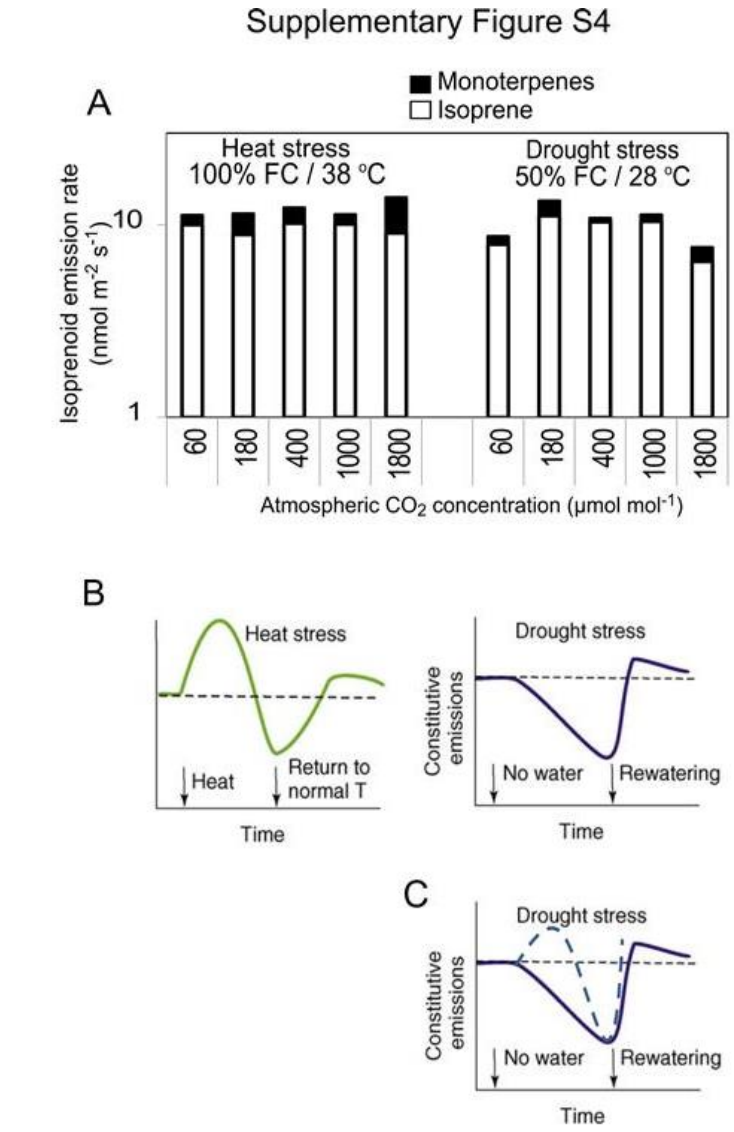

Fig. S4: Compare emissions under heat (without drought) and drought (without heat)

Fig. S4: **Isoprene and monoterpene emission rates: Comparing drought and heat responses** (A) Compare emissions under heat (without drought) and drought (without heat) (B) Illustration reproduced (permission pending) from Niinemets (2010) in Trends in Plant Science (C) We propose that the response of constitutive isoprenoid emission under drought follows the blue dashed line rather than the original solid line (compare with heat stress response)

Supplementary Figure S5

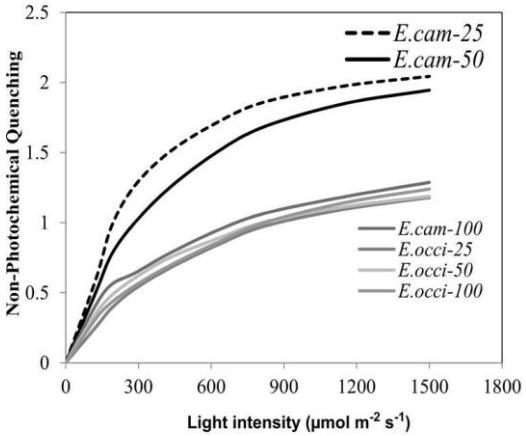

Fig. S5: Estimated NPQ across drought gradient

Fig. S5: Estimated NPQ across drought gradient (see main MS methods for details) Note the significantly high NPQ in *E. camaldulensis* subsp. *camaldulensis* (%FC <50)

Supplementary Figure S6

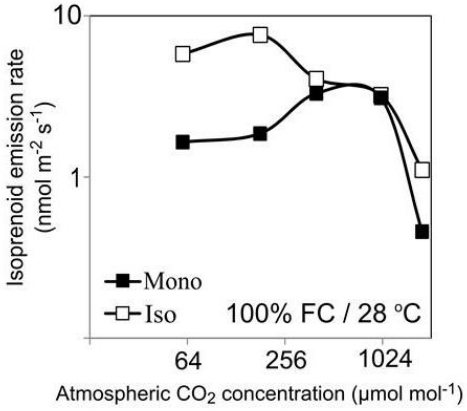

Fig. S6 Isoprene and monoterpene emission rates peaking at two different CO_2 concentrations

Fig. S6: **Isoprene and monoterpene emission rates peaking at two different CO_2 concentrations** in response to short term CO_2 exposure (at 28 ºC, 20% O_2) in *E. camaldulensis* subsp. *obtusa*

Seasonal rhythm of volatile isoprenoid emission in eucalypts is entrained by temperature and is photoperiod-gated

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Table S1: Primers for real time qPCR

Target cDNA	Original Sequence Source	Primer Name	Sequence (5' to 3')	GC (%)	T_m (°C)	Amplicon Length (bp)	qPCR efficiency	Pathway
DOXP synthase[1] 1-deoxy-D-xylulose 5-phosphate synthase	EGB[2] and MSA[3]	DXS FP	ATT CAT GCT GCG ATG GGA GG	55.0	57.8	**426**	ND	The MEP pathway
		DXS RP	GAA CGC CAA CTC CAT TTC CTC	52.4	56.3			
HMD reductase 4-hydroxy-3-methylbut-2-enyl diphosphate reductase	*Arabidopsis thaliana* and EGB	HDR FP	GAA CTC AAG CAA CAC ATC TCA CCT TCA A	42.9	59.1	**225**	1.89	
		HDR RP	CCT TCA CTA AAG CAT CCT CAA CGA CC	50.0	59.3			
Isoprene synthase[4]	*Populus* spp (MSA)	ISPS FP	TGG ATG AGT TGG AGC TAT TTA CAG ATG C	42.9	58.5	**550**	1.85	
		ISPS RP	GTT CAT CTT TTT CCA GGT TTC GTC GAT C	42.9	58.0			
Monoterpene synthase	*Eucalyptus globulus*	MTS FP	CGG TCT TTA TGA GCC TAC AGA AGT C	48.0	56.8	**360**	1.99	
		MTS RP	TTC GAG AAT TTG AGG ATT CGT GTA TTC	37.0	55.3			
ATP synthase[5]	*Chlamydomonas reinhardii* *Zea mays* and EGB	ATP FP	GTC AGC GTG GGG AAG AAG G	63.2	58.3	**182**	1.91	Light reactions (Energetics)
		ATP RP	ACG AAC TTG GTG TAG AGG AGC	52.4	56.6			
Fd-NADP$^+$ oxidoreductase[6]	*Spinacia oleracea* *Oryza sativum,* EGB	FNR FP	GCG ACA ATT ATC ATG CTT GCC ACC	50.0	59.1	**210**	ND	
		FNR RP	CTT CTC ATT CGT TTG CTC TCG GCT C	52.0	59.7			
Ribulose bis phosphate carboxylase oxygenase (SSU)[7]	*Eucalyptus globulus*	RBC SSU FP	AGA CTC TCT CCT ACC TTC CAC	52.4	55.0	**300**	1.96	Photosynthetic Carbon reduction
		RBC SSU RP	GCA TTG CAC TTG ACG CTT G	52.6	55.5			
Phosphoenol pyruvate carboxylase (PEPCase)	EGB	PEPC FP	GAA CTG TTG GAA GAG GAG GTG	52.4	55.2	**400**	2.10	Link with photosynthesis & MEP pathway
		PEPC RP	GTC TGA GTC CAT GCG AAG ATC	52.4	55.3			
Actin	*Eucalyptus grandis*	ACT FP	ACT GGA ATG GTG AAG GCT GG	55.0	57.5	**400**	1.99	qPCR control
		ACT RP	GTT GTA CGA CCA CTG GCA TAA AGG	50.0	57.9			
18S ribosomal RNA gene	MSA	18S FP	CCA AGG AAG TTT GAG GCA ATA ACA GGT CT	44.8	60.4	**260**	1.97	
		18S RP	CAT TCA ATC GGT AGG AGC GAC GG	56.5	59.7			

[1] Primers spanning exon 7 and 8 with an intron ~100bp; [2] *Eucalyptus* genome BLAST; [3] Multiple sequence alignment; [4] Primers spanning exon 4 and 7 including three introns of 100bp or more
[5] Primers spanning exons 4 and 5 including the largest intron of 700bp (chloroplastic γ-subunit); [6] Primers are partially unique to chloroplastic Fd-NADP$^+$ Reductase; [7] Rubisco small sub-unit

Figure S1: Plant height and heterophilly in *E. globulus*

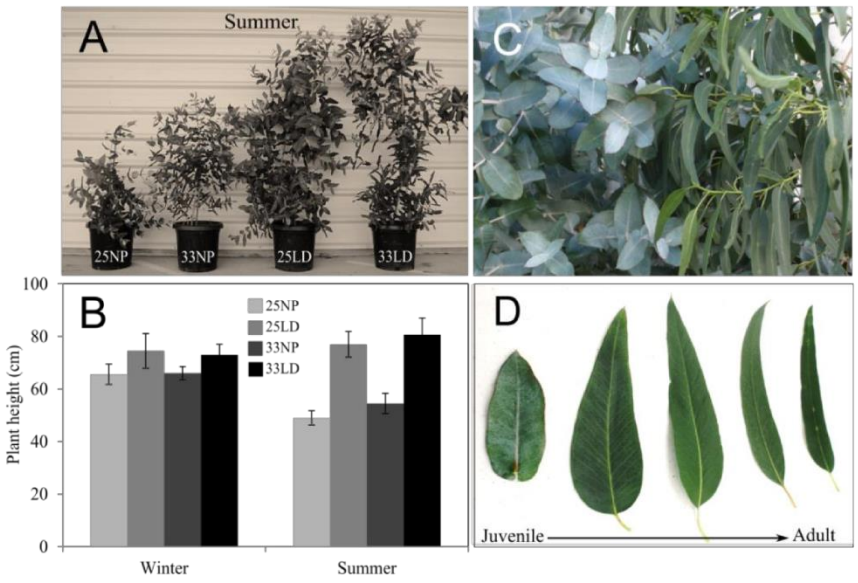

Figure S1: Plant height and heterophilly in *Eucalyptus globulus*: (A) Photograph showing the positive effect of LD acclimation in summer on stem elongation and vegetative growth independent of temperature acclimation (B) Actual data of plant heights in winter-ascent (differences not significant) and summer-ascent (significant) (C) Morphological difference between juvenile and adult plants of *E. globulus* with (D) showing transitory stages of development.

Figure S2: Long day and low temperature detrimental impact on *E. globulus*

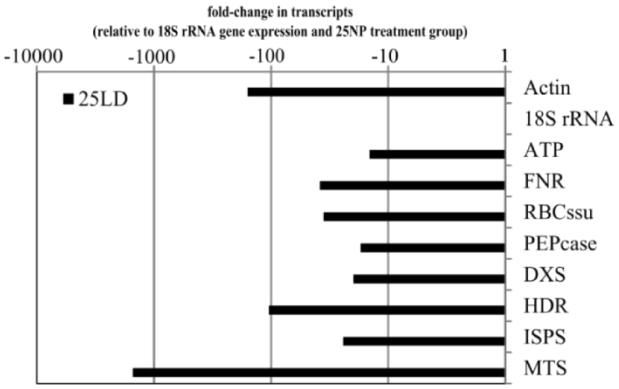

Figure S2: Exceptional detrimental impact of LD on *Eucalyptus globulus* at 25 ˚C: *E. globulus* at 25 ˚C acclimated to LD showed hundreds of folds of decline in transcripts of all genes including actin, which was originally thought of as a reference gene. Since the treatment caused a detrimental change in the physiological status of *E. globulus*, their photosynthetic and volatile emission response were not explainable within the experimental context.

Table S2: Photosynthesis in *E. camaldulensis* during winter-ascent						
Treatment group		Net assimilation rate (μmol $m^{-2}s^{-1}$)	Stomatal conductance (mol $m^{-2}s^{-1}$)	C_i at C_a= 400 μmol mol^{-1}	Transpiration rate (mmol $m^{-2}s^{-1}$)	Relative Humidity (%)
25NP	Mean	15.5	0.300	272	5.3	43
	Std. Err	1.36	0.054	12	0.63	2
25LD	Mean	14.6	0.228*	256	4.6	42
	Std. Err	1.02	0.032	14	0.48	2
33NP	Mean	18.5	0.330	252	7.3	44
	Std. Err	1.01	0.047	19	0.65	3
33LD	Mean	15.1	0.243**	255	6.2	35
	Std. Err	0.81	0.031	21	0.48	2
* P=0.11; **P=0.04						

Appendix VI

Supplementary information for Chapter 6

Supplementary Material

Evolution of isoprene emission capacity in plants

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4 Supplementary tables

Summary of additional anecdotal evidence

85 Supplementary references

Table S1: Net assimilation rate , isoprene and monoterpene emission rates for selected species of angiosperms (data points superimposed on to the conceptual diagram in Figure 2b of the main paper)

Plant	Photosynthetic rate (μmol/m^2/ s)	SE	Isoprene emission rate (nmol/m^2/s)	SE	Refs
Populus trichocarpa	18.73	1.04	45.21	8.85	[S1]
	20 [a]	*	45 [a]	*	[S2]
Populus deltoides	18.6	0.8	40.4	3.4	[S3]
	14.86	1	21.4	4.17	[S4]
Populus euramericana	16.3	1.22	24.99	5.53	[S1]
	15.45	1.79	24.99	5.07	
	15.86	1.41	28.24	4.8	
Populus nigra	13.36	1.14	20.13	3.99	[S1]
	15.51	1.74	24.86	4.13	
	14.03	1.17	34.78	9.37	
	20.19	0.86	20.98	1.41	
	14.66	1.59	18.18	5.96	
	14.79	0.4	37.86	3.33	
	15 [a]	*	20 [a]	*	[S5]
	16.13	0.77	19.47	0.85	[S6]
Populus alba	17.58	1	15.39	1.36	[S7]
Populus canescence	17 [a]	*	33 [a]	*	[S8]
Quercus rubra	9.3	1.4	29.8	4.6	[S9]
	14.5	0.9	45.7	2.8	
	9.9	0.5	43.6	8.3	
	11.2	0.4	76.8	8.8	
	9 [a]	*	38 [a]	*	[S10]
Quercus robur	3.5 [a]	*	14 [a]	*	[S11]
	8 [a]	1	18 [a]	1	[S12]
	8 [a]	2	30 [a]	5	[S13]
Quercus alba	11.2	0.4	87	9	[S14]
Quercus petraea	6.6	2.5	4.3	2	[S15, S16]
Quercus frainetto	8 [a]	*	30.7	*	
Quercus pubescence	11.9 [a]	4	28 [a]	0.5	[S17]
Eucalyptus viminalis	5.2 [a]	0.2	0.84 [a]	0.59	[S18]
Eucalyptus grandis	3 [a]	0.1	2.58 [a]	1.37	
Eucalyptus camaldulensis	6.16 [a]	0.31	5.05 [a, b]	3.56	[S18]
Eucalyptus globulus (adult)	8.2 [a]	0.44	11.86 [a]	0.56	
	11 [a]	1.5	17.2	*	[S19]
	20 [a]	1.5	22 [a, c]	2	[S20]
Eucalyptus globulus (juvenile)	9.4	1.4	8.2 [a]	1.4	[S21]
	7.5	0.8	6.9 [a]	0.8	

[a] taken from a graph; [b] PPFD not at saturating level; [c] monoterpenes not reported; * NA

All chosen isoprene emission rates for angiosperms also reported simultaneously measured A_{sat} and the conditions were CO_2 between 350 to 400 μmol/mol, temperatures of 25 to 30 °C, and PAR in the range of 500 to 1000 μmol/m^2/s. Due care is given to the measurement season, temperature and light intensity levels when choosing values for comparison.

Table S2: Net assimilation rate , monoterpene emission rates for selected species of evergreen gymnosperms (data points superimposed on to the conceptual diagram in Figure 2b of the main paper)

Plant	A_{sat} (as reported)	A_{sat} (units)	Refs.	Monoterpene emission rate (M_e)	M_e (units)	Refs	SLA (cm^2/g)	reference	A_{sat} ($\mu mol/m^2/s$)	M_e ($nmol/m^2/s$)
Pinus pinea	2.5	$\mu mol/m^2/s$	[S22]	23	$\mu g/g/hr$	[S22]	25	[S23]	2.5	18.88
Pinus pinea	5	$\mu mol/m^2/s$	[S22]	2.1	$\mu g/g/hr$	[S22]	25	[S23]	5	1.67
Pinus pinea	4	$\mu mol/m^2/s$	[S22]	7.6	$\mu g/g/hr$	[S24]	25	[S23]	4	6.22
Pseudotsuga menziesii	10±2.5	$\mu mol/m^2/s$	[S25]	22	$\mu g/g/hr$	[S25]	70	[S26]	10	6.34
Pseudotsuga menziesii	0.02	umol/g/s	[S27]	10.5	$\mu g/g/hr$	[S27]	70	[S26]	6.67	3.17
Pinus ponderosa	0.03	umol/g/s	[S27]	3	$\mu g/g/hr$	[S27]	20	[S28]	6.9	4.17
Pinus massoniana	10±5	$\mu mol/m^2/s$	[S29]	2400	$\mu g/g/hr$	[S29]	50	[S30]	10	1.25
Picea abies	70±10	$\mu mol/g/hr$	[S31]	0.1 to 0.5	$\mu g/g/hr$	[S31]	53	[S32]	3.58	0.11
Pinus banksiana	13.4	$\mu mol/m^2/s$	[S33]	1.25±0.25	$\mu g/g/hr$	[S34]	56	[S35]	13.4	0.5
Pinus sylvestris	2.2	$\mu mol/m^2/s$	[S36]	100±10	$ng/m^2/s$	[S37]	43	[S38]	2.2	0.78
Pinus halepensis	8±2	$\mu mol/m^2/s$	[S39,S40]	0.13	$\mu g/g/hr$	[S24]	52	[S41]	8	0.05
Pinus halepensis	8±2	$\mu mol/m^2/s$	[S39,S40]	2	$\mu g/g/hr$	[S42]	52	[S41]	8	0.8
Abies religiosa	3.5 to 12.96	$mg/dm^2/hr$	[S43]	6	$\mu g/g/hr$	[S44]	NA	[S45]**	2	3.05
Pinus taeda	6	$\mu mol/m^2/s$	[S46]	100 to 250	$\mu g/m^2/hr$	[S47]	NA	NA	6	0.41
Pinus elliottii	5±1	$\mu mol/m^2/s$	[S48]	250	$\mu g/m^2/hr$	[S47]	NA	NA	5	0.51
Pinus resinosa	2.5	$\mu mol/m^2/s$	[S49]	11	$\mu g/g/hr$	[S50]	65	[S49]	2.5	3.45
Abies lasiocarpa	2	$\mu mol/m^2/s$	[S51]	16	$\mu g/g/hr$	[S50]	40	[S45]	2	8.16
Thuja occidentalis	4	$\mu mol/m^2/s$	[S52]	9.8	$\mu g/g/hr$	[S50]	77	[S53]	4	2.59

For gymnosperms, A_{sat} and emission rates were not measured simultaneously (except a few) hence A_{sat} values were sourced from alternate references. A_{sat} values were also calculated on a leaf area basis by using Specific Leaf Area (SLA) estimates for the respective species obtained from the literature. Therefore, the relationship between assimilation rate and isoprenoid emission rate in gymnosperms should be treated only as indicative and not as absolute.
**SLA of *Abies lasiocarpa*

Table S3: Net assimilation rate, isoprene (+monoterpene) emission rates for selected species of evergreen and deciduous gymnosperms (data points superimposed on to the conceptual diagram in Figure 2b of the main paper)

Plant	A_{sat} (as reported)	A_{sat} (units)	Refs.	Iso + mono emission rate	Units	Refs.	SLA (cm^2/g)	Refs.	Asat ($\mu mol/m^2/s$)	Isoprene + monoterpene emission rate ($nmol/m^2/s$)
Picea sitchensis	100±20	µmol/g/hr	[S54, S55]	20±8	µg/g/hr	[S56]	40	[S26]	5	1.51
Picea sitchensis	5	$\mu mol/m^2/s$	[S54, S55]	0.48	$nmol/m^2/s$	[S57]	40	[S26]	5	0.48
Pinus sylvestris	2.2	$\mu mol/m^2/s$	[S36]	0.63	$nmol/m^2/s$	[S36]	NA	-	2.2	0.63
Larix decicua	2.4	$\mu mol/m^2/s$	[S36]	3.05	$nmol/m^2/s$	[S36]	NA	-	10.5	3.05
Picea abies	4.5	$\mu mol/m^2/s$	[S36]	1.18	$nmol/m^2/s$	[S36]	NA	-	4.5	1.18
Taxodium distichum	6.9	$\mu mol/m^2/s$	[S58]	3.38	µg/g/hr	[S59]	50	[S60]	6.9	1.55
Taxodium ascendens	11.9	$\mu mol/m^2/s$	[S61]	3.95	µg/g/hr	[S59]	50	[S60]	11.9	1.8
Pinus massoniana	15	$\mu mol/m^2/s$	[S30]	23.4	µg/g/hr	[S59]	50	[S30]	15	13.88
Picea engelmannii	3.4	$\mu mol/m^2/s$	[S62, S63]	14	µg/g/hr	[S64]	64	[S65]	3.4	9.54
Picea glauca	13.4	$\mu mol/m^2/s$	[S66]	16.26	µg/g/hr	[S66]	67	[S67]	13.4	9.53
Picea pungens	13	$\mu mol/m^2/s$	[S66]	13.01	µg/g/hr	[S64]	67	[S67]	13	7.26
Picea abies	4.5	$\mu mol/m^2/s$	[S36]	3.49	µg/g/hr	[S64]	53	[S68]	4.5	1.46
Metasequoia glyptostroboides	8	$\mu mol/m^2/s$	[S69]	20	µg/g/hr	[S50]	145	[S70]	8	3.25
Pteridophytes (isoprene emitters)										
Thelypteris kunthii	6.8	$\mu mol/m^2/s$	[S71]	10.5	$nmol/m^2/s$	[S71]	NA	-	6.8	10.5
Dicksonia antarctica	3	$\mu mol/m^2/s$	[S71]	10.8	$nmol/m^2/s$	[S71]	NA	-	3	10.8
Thelypteris decursive-pinnata	3.5	$\mu mol/m^2/s$	[S71]	13.5	$nmol/m^2/s$	[S71]	NA	-	3.5	13.5

For gymnosperms, A_{sat} and emission rates were not measured simultaneously (except a few) hence A_{sat} values were sourced from alternate references. Therefore, the relationship between assimilation rate and isoprenoid emission rate in gymnosperms should be treated only as indicative and not as absolute.

Anecdotal evidence:

(a) When examined further, isoprene emission appears to have evolved even in less diverse trees such as *Pseudolachnostylis maprouneifolia*, a high isoprene emitting, monotypic tree genus from the African savannah [S72], which belongs to Phyllanthaceae, a family comprising > 1200 species within the non-isoprene emitting, mostly herbaceous genus *Phyllanthus* [S73]. It has been estimated that speciation rates in herbaceous plants (1.15 species/lineage/10^6 yr) are nearly ten times greater than in trees (0.12 sp/lin/10^6 yr) [S74]. A large contingent of herbaceous genera in a plant family would probably mean a higher growth and speciation rate in their nearest woody relatives. Arborescent lycophytes from the Carboniferous period such as *Lepidodendron*, and horsetails such as *Calamites* which grew up to 30 m in height [S75], were also potential isoprene emitters. The rates of increase in speciation and complexity in the palaeo-ecosystems of Late Devonian (400 MYa) and Carboniferous (300 MYa) are estimated to be among the highest in the geological history of land plants [S76].

(b) Evergreens *Quercus ilex* and *Q. suber* are monoterpene emitters while the deciduous *Q. robur* and semi-evergreen *Q. canariensis* are isoprene emitters (e.g. [S77]). Evergreen habit-monoterpene emission vs. Deciduous habit-isoprene emission, the combinational co-occurrence needs to be examined further. Deciduous eucalypts (*Eucalyptus alba,* and *E. grandifolia*) from the northern subtropical forests in Australia would be good candidates to test the hypothesis that the pattern in leaf life span influence on emission capacity is conserved, even in eucalypts that emit both isoprene and monoterpenes.

Table S4: Wilcoxon's two tailed signed-ranks test performed with collective number of species for emitting and non-emitting sister genera treated as paired observations (references for original phylogenies are given in parenthesis)

	Isoprene non-emitters	Number of species	Isoprene emitters	Number of species	Difference	Rank
Fagaceae [S78]	*Fagus*	11	*Quercus*	605		
	Lithocarpus	336				
	Castanea	9				
	Castanopsis	137				
		493		***605***	**112**	**4**
Fabaceae [S79, S80]	*Cercis*	***10***	*Bauhinia*	***352***	**342**	**10**
	Cassia	87	*Caesalpinia*	163		
	Ceratonia	2	*Acacia*	1380		
			Albizia	138		
		89		***1681***	**1592**	**14**
	Phaseolus	***87***	*Dalbergia*	***278***	**191**	**7**
Salicaceae [S81, S82]	*Chosenia bracteosa*	***1***	*Salix*	***377***		
	Azara	9	*Populus*	87		
	Idisea polycarpa	1				
		11		***464***	**453**	**11**
Myrtaceae [S83]	*Allosyncarpia ternata*	1	*Corymbia*	95		
	Stockwellia quadrifida	1	*Angophora*	16		
	Eucalyptopsis	2	*Eucalyptus*	810		
	Arillastrum gummiferum	1				
		5		***921***	**916**	**13**
Gymnospermae [S84]	*Juniperus*	71	*Metasequoia*	2		
	Callitris	16				
	Cryptomeria japonica	1				
		88		***2***	**-86**	**-1**
	Nageia	***6***	*Podocarpus*	***108***	**102**	**3**
	Tsuga	10	*Pinus*	122		
			Picea	40		
			Larix	14		
		10		***176***	**166**	**6**
Pteridophyta [S85]	*Dryneria*	***16***	*Polypodium*	***160***	**144**	**5**
	Rumohra	***3***	*Dryopteris*	***286***	**283**	**8**
	Blechnum	97	*Thelypteris*	383		
			Cyclosorus	170		
		97		***553***	**456**	**12**
	Pteris	***149***	*Adiantum*	***126***	**-23**	**-2**
	Marselia	***12***	*Cyathea*	***300***	**288**	**11**

The calculated sum of negative ranks is 3 and it is less the table value of 12 (when N=14) for Wilcoxon singed ranks test at ***P<0.01.*** Thus, the hypothesis that isoprene emission occurs in genera that are significantly more speciose than their nearest sister genera is accepted (P<0.01).

Absolute sum of positive ranks	**102**
Absolute sum of negative ranks	**3**

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Appendix VII

ADDENDUM

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Species-specific photorespiratory rate, drought tolerance and isoprene emission rate in plants

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The effect of drought on plant isoprene emission varies tremendously across species and environments. It was recently shown that an increased ratio of photosynthetic electron transport rate (ETR) to net carbon assimilation rate (NAR) consistently supported increased emission under drought. In this commentary, we highlight some of the physiological aspects of drought tolerance that are central to the observed variability. We briefly discuss some of the issues that must be addressed in order to refine our understanding of plant isoprene emission response to drought and increasing global temperature.

Keywords: drought, electron transport rate, isoprene emission, MEP pathway, photorespiration

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Increasing and irregular droughts induced by global warming will affect plant responses and could potentially define land plant evolution during the Anthropocene.[1] Terrestrial plant ecosystems are a major sink for CO_2 and constitute a central component of global carbon cycle. Net primary production (NPP; ~50 PgC/yr) is sensitive to changes in global temperatures and could decline in future due to unprecedented warm temperatures and extended periods of drought.[2-3] The emission of isoprene (~0.5 PgC/yr) by forests releases carbon to the atmosphere in a form that influences oxidation reactions in the troposphere.[4] Emissions increase in plants under oxidative stress caused by high light, low CO_2, drought and heat; such observations have led to various hypotheses concerning the biological function and evolutionary role of isoprene emission in ecosystems.[5-8] While photosynthesis clearly declines under abiotic stresses (particularly drought), isoprene emission responses are far less predictable.[9]

Isoprene biosynthesis and emission by plants depends on carbon, energy and reducing power supplied by photosynthesis.[10-12] One of the hypotheses that attempts to explain variation in isoprene emission states that secondary metabolism and volatile emissions increase under abiotic stresses because the net carbon assimilation declines but the supply of reducing equivalents remains high.[13-15] We have successfully tested the hypothesis that photosynthetic carbon reduction competes with non-photosynthetic sinks for reducing power under stress-free conditions. A claim is made that residual reducing power unused by carbon assimilation drives increased isoprene emission under various abiotic stresses[16,17] (**Fig. 1**).

Exploiting the contrasting drought tolerance of 2 *Eucalyptus* species, we provided[16] an exhaustive experimental data-set showing that an increased ETR-to-NAR ratio sustained increased isoprene emission rates under drought, which appears to follow a species-specific threshold for drought tolerance (**Fig. 1**). When placed in an ecological context, aggregate isoprene emission depends on the mix of species in plant communities, each emitting at a characteristic range of rates.[13] While vegetation maps define species distributions reasonably well, there is still very scarce information about the physiological determinants of emission rates. Improving global emission algorithms requires a broad consensus of species' responses to drought, which so far has proven a challenge. In this addendum to

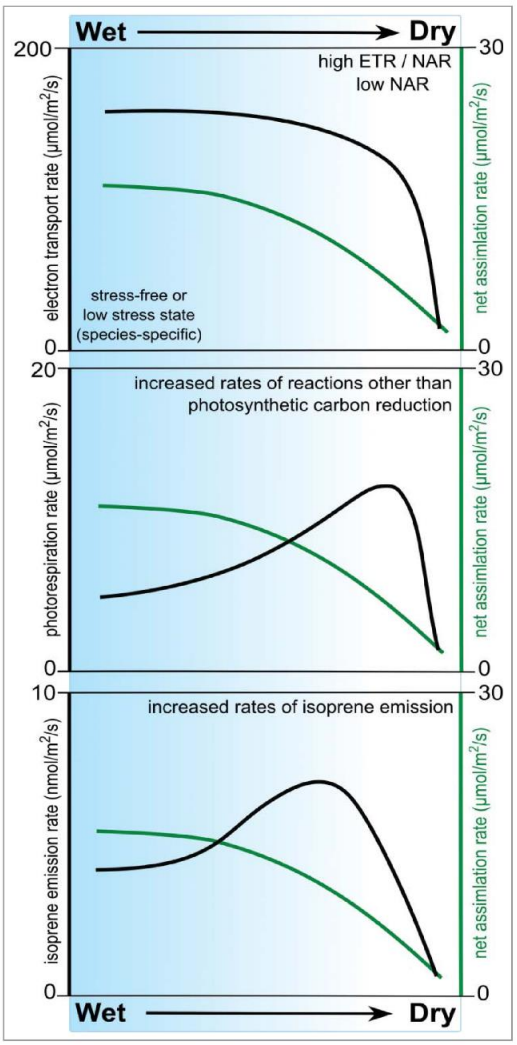

Figure 1. A notional view of the relationship between net assimilation rates (green line in all images) and electron transport rate, photorespiration rate and isoprene emission rate (top to bottom) as eucalypts are to exposed diminishing water supply.

the paper by Dani et al.,[16] we highlight issues that are yet to be addressed.

We have monitored photosynthesis (at leaf-level) and isoprene emission (both at leaf and whole plant-level) under glasshouse conditions in many species of genetically diverse eucalypts selected from different regions across Australia. While any relationship between emission rates obtained through canopy sampling (of young seedlings) and leaf-level photosynthesis gas exchange measurements has epistemological problems, and is at best only indicative, we wish to highlight some pertinent observations that we believe are relevant to future studies involving isoprene emission and abiotic stress.

The emission rates observed in all of our experiments involving eucalypts, including both multiple species and the pairwise comparison cited above,[16] were low (0 to 10 nmol/m^2/s) compared with those from more widely studied poplars and oaks (20 to 100 nmol/m^2/s).[6] Many native Australian plants have abundant Rubisco and eucalypts in particular are notable for their extremely high photosynthetic capacities among trees, operating as they generally do in high-light environments.[18] While a large carboxylation capacity could make full use of high energy status of the leaves of eucalypts, it could also leave limited reducing power for the MEP pathway under stress-free conditions. These characteristics, along with perennial monoterpene storing, may explain why evergreen eucalypts emit less isoprene than deciduous poplars or oaks, which go through annual cycles of senescence and periodic drought. A strong positive correlation between photosynthetic electron transport and isoprene emission is known[19] and based on published recent empirical data[16,17] we project that plants with low photosynthetic rates should be high isoprene emitter if they posses high electron transport rates relative to carbon assimilation rates.

CO_2 compensation point (Γ^*) is an important indicator of photorespiratory capacity of a species and it takes an appreciable range of values even among C_3 plants (30 to 70 ppm).[20] Drought exacerbates oxidative stress by decreasing stomatal conductance and as a result increasing leaf temperature and CO_2 compensation point. Γ^* also conforms to a diurnal cycle (40 ppm to 160 ppm), with a daily maximum coinciding with midday depression in carboxylation efficiency.[21] The well-known diurnal peak of isoprene emission just after midday is consistent with the mechanistic link between carboxylation efficiency and emission rates. We also observed a large range in Γ^* values (40 ppm to 80 ppm) across many species of the genus *Eucalyptus* under stress-free conditions. This diversity in photorespiration among eucalypts has important implications for the way drought-induced photorespiration affects isoprene emission in trees. Photorespiration directly competes with the MEP pathway for reducing power and the competition becomes acute under drought stress as photorespiration rate increases and carbon supply for the MEP pathway decreases.[16] The large

range in Γ* could be due to diversity in leaf structural traits (mesic vs. xeric) that in turn reflect rainfall and nutrient availability in their respective habitats. Many C_3 plants are known to recapture photorespiratory CO_2 and the energy requirements of such processes are completely unknown.

It is recently reported that drought-induced increase in concentration of soluble sugars in the cytoplasm has no bearing on isoprene emission.[22] These findings rule out import of cytosolic carbon (sugars) into chloroplasts under severe drought. It further reinforces the claim that isoprene emission behavior is most strongly influenced by photorespiration and other pathways occurring *within* chloroplasts, which therefore compete directly with the co-located MEP pathway for both photosynthetic carbon and reducing power.

The interactive effects of CO_2, heat and drought on emission are quite complex.[16] Even closely related eucalypts exhibit significant differences in their photorespiratory sink strengths for reducing power, indicating that differences between more distantly related taxa could be even more striking and could make the assessment of their emission responses to drought far more difficult using existing models and mechanistic assumptions. Elevated CO_2 levels for long periods are suggested to cause a significant decrease in maximum carboxylation capacity in many C_3 plants (due to less Rubisco[23]), although the response of electron transport rate is less clearly defined.[24] Acclimation of plants to elevated CO_2 and its impact on carbon gain, plant water use efficiency uncertain.[25] Changes in carboxylation and RUBP regeneration (contents of chlorophyll and Rubisco)[26] cannot be generalized across many taxa that characterize important isoprenoid-emitting ecosystems. Our results suggest that obtaining a reliable global atlas of tolerances for abiotic stresses among major plant biomes is crucial to understanding the future of plant isoprene emission in a high-CO_2 world.

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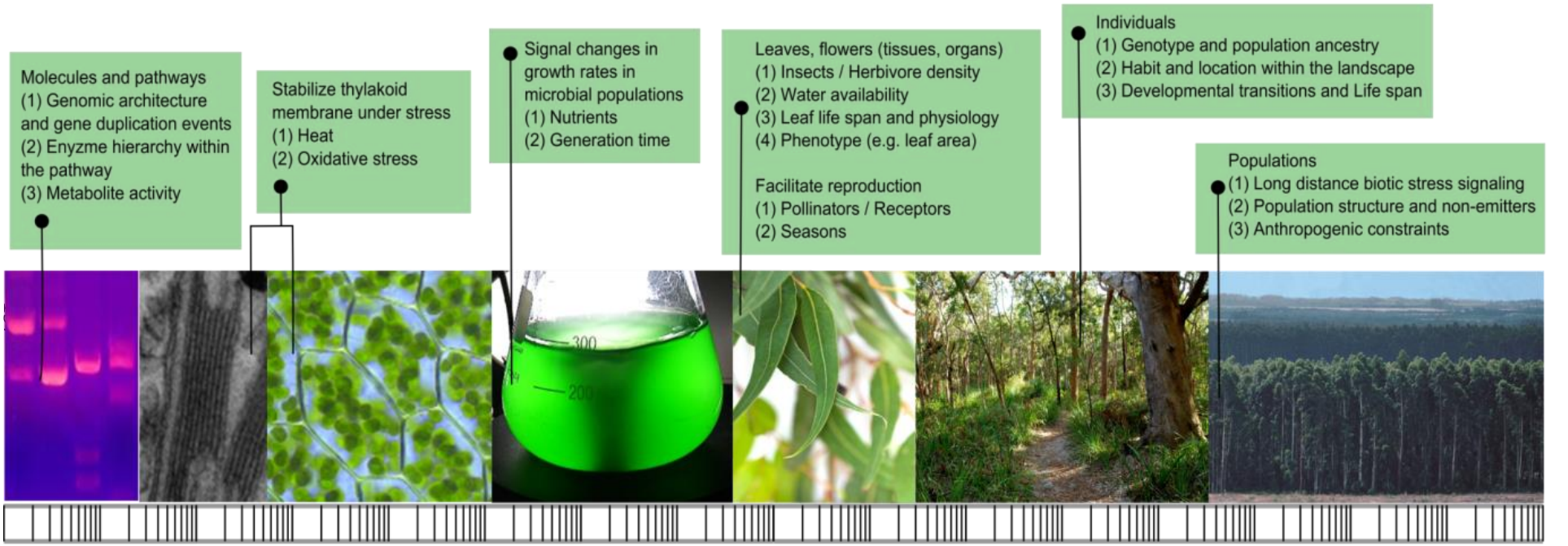

Function of volatile isoprenoids (isoprene and monoterpenes) and means of natural selection