Methoxyl groups of plant pectin as a precursor of atmospheric methane: evidence from deuterium labelling studies

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Summary

• The observation that plants produce methane (CH_4) under aerobic conditions has caused considerable controversy among the scientific community and the general public. It led to much discussion and debate not only about its contribution to the global CH_4 budget but also about the authenticity of the observation itself. Previous results suggested that methoxyl groups of the abundant plant structural component pectin might play a key role in the *in situ* formation process of CH_4. Here, this effect is investigated using an isotope labelling study.

• Polysaccharides, pectin and polygalacturonic acid, with varying degrees of trideuterium-labelled methyl groups in the methoxyl moieties, were investigated for CH_4 formation under UV irradiation and heating.

• A strong deuterium signal in the emitted CH_4 was observed from these labelled polysaccharides.

• Results clearly demonstrate that ester methyl groups of pectin can serve as a precursor of CH_4, supporting the idea of a novel chemical route of CH_4 formation in plants under oxic environmental conditions.

Key words: climate, greenhouse gas, stable hydrogen isotopes, sugars, vegetation.

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Introduction

It is well known that plants emit a wide range of volatile organic compounds (VOCs), such as isoprenoids and oxygenated compounds (e.g. methanol and acetone), to the atmosphere (Kesselmeier & Staudt, 1999). However, it was only recently found that plants also produce CH_4 when we showed that intact living plants, as well as plant litter, produce this important greenhouse gas in an oxygen-rich environment and release it to the atmosphere (Keppler *et al.*, 2006). We also reported that CH_4 emissions were sensitive to both temperature and natural sunlight irradiation. The fact that plants produce CH_4 under aerobic environmental conditions is a surprising finding because, before our observations, biological formation was only considered to occur by microbial activity under strictly anaerobic conditions, in environments such as wetlands rice paddies, or the intestinal tract of ruminants. Our controversial findings have led to considerable discussion and debate as to their authenticity and their implications for the CH_4 global budget and global warming (Kirschbaum *et al.*, 2006; Schiermeier, 2006; Dueck *et al.*, 2007; Evans, 2007). Most importantly, Dueck *et al.* (2007), utilizing ^{13}C-labelled plants, reported that there were no substantial emissions of CH_4 from living plants, which obviously casts serious doubt on our work. However, on the other hand, a very recent study by Wang *et al.* (2008) reported emissions of CH_4 from several shrubs of the Mongolian steppe, confirming the finding of aerobic methane formation in plants.

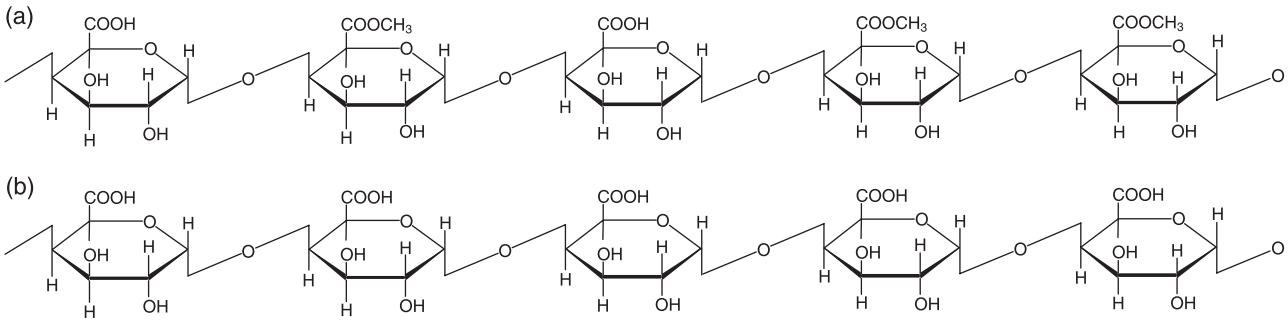

Fig. 1 Chemical structure of pectin (a) and polygalacturonic acid (PGA) (b).

Interestingly, again very recently, via a personal communication, we became aware of work conducted in the late 1950s at the Academy of Sciences of Georgia (Tbilisi) on emissions of VOCs from leaves of *Populus simonii* Carr. and *P. sosnowskyi* Grossh (Sanadze & Dolidze, 1960). In that study, the researchers suggested that plants could emit CH_4 as well as ethane, propane, isoprene and several other volatile organic components. However, no follow-up studies on CH_4 release were undertaken, as this group very successfully focused on isoprene emissions instead. Even though this early report and the more recent space, aircraft and surface observations, suggesting a strong CH_4 source in tropical forest regions (Frankenberg *et al.*, 2005; Crutzen *et al.*, 2006; Bergamaschi *et al.*, 2007; Miller *et al.*, 2007; Sinha *et al.*, 2007), together with the Wang *et al.* (2008) study provide some support for our contention that vegetation releases CH_4, the mechanism of its formation remains unknown. Elucidation of the underlying pathway for aerobic CH_4 is crucial to enable a full understanding of its role and hence its global significance.

As a first step, it is important to gain information about precursor compounds in plants that could give rise to CH_4. Based on previous results we suggested the possibility of the involvement of the methyl moiety of the esterified carboxyl group (methoxyl group) of pectin (Keppler *et al.*, 2004). Indeed, in experiments with apple pectin we not only observed emission of CH_4 but also noted that the emission rate was broadly similar to that measured with detached leaves (Keppler *et al.*, 2006). However, even though those results indicated a role for pectin, they provide no proof for the involvement of the pectin methoxyl group in CH_4 formation.

Stable isotope analysis is a powerful tool that we recently employed to demonstrate that plant pectin and lignin methoxyl groups have unique carbon isotope signatures (Keppler *et al.*, 2004), and also to establish the relationship between hydrogen isotopes of wood lignin methoxyl groups and meteoric water (Keppler *et al.*, 2007). In this investigation we again employ this technique, together with pectin and polygalacturonic acid (PGA) (Fig. 1), modified to contain varying degrees of methoxyl moieties with varying degrees of trideuterium-labelled methyl groups, to demonstrate that plant pectin methoxyl groups are a precursor of CH_4 and thus could be a source of it in an oxic environment.

Materials and Methods

Isotopic labelling of pectin and polygalacturonic acid

All reagents were purchased from Sigma-Aldrich Company Ltd except for low methoxyl pectin (GENU® pectin LM-101 AS), which was a gift from CP Kelco Ltd (Lille Skensved, Denmark) and methyl D-galactopyranoside which was purchased from CMS Chemicals (Abingdon, UK).

Methyl esterification

Methyl esterification was performed essentially as described by van Alebeek *et al.* (2000). Briefly, samples of polygalacturonic acid (2 ± 0.01 g) were weighed into 100 ml volumetric flasks and solutions of anhydrous methanolic H_2SO_4 (0.02 N, 100 ml) containing either 0, 5 or 20% tetradeuterated methanol (v/v) were added. Samples were incubated at 4°C for 47 d with occasional shaking and then centrifuged (770 *g*, 10 min). The methanolic H_2SO_4 was decanted and the remaining sample washed with propan-2-ol (3×40 ml), with the supernatant being discarded following centrifugation. The samples were left overnight at room temperature to allow evaporation of the remaining propan-2-ol, and then distilled water (20 ml) was added to the samples, which were then thoroughly mixed, frozen and lyophilized over a 6 d period. Dried samples were ground to a powder using a pestle and mortar. Low methoxyl pectin was methylated by the procedure described earlier, except that only 5% tetradeuterated methanol was used for derivatization and the incubation period was 14 d.

Determination of deuterium label in pectin and polygalacturonic acid

Methoxyl content was determined by measuring the release of methyl iodide (CH_3I) using the Zeisel technique as described

Table 1 Methoxyl content and % trideutered methyl groups in methyl-esterified polygalacturonic acids and pectin

Sample	Methoxyl content (%)	% Labelled methoxyl groups	Theoretical $\delta D(CH_4)$ (‰ vs VSMOW)*
Polygalacturonic acid esterified with unlabelled methanol	1.44	–	–
Polygalacturonic acid esterified with 5% CD_3OD labelled methanol	1.48	6.1	312 000
Polygalacturonic acid esterified with 20% CD_3OD labelled methanol	2.30	22.6	1 410 000
Pectin treated with 5% CD_3OD labelled methanol	4.08	0.05	1 660

*Calculated using the following equation: δ^2H (‰) = (($^2H/^1H_{methoxyl} \times 0.75$) + ($^2H/^1H_{standard} \times 0.25$) – $^2H/^1H_{standard}$))/$^2H/^1H_{standard} \times 1000$‰ and the assumption that for the four hydrogen atoms of the formed CH_4, three hydrogen atoms (75%) are derived from the methoxyl group (OCH_3) and one (25%) comes from either surrounding water or the organic model compound with a theoretical value of δ^2H of 0‰ ($^2H/^1H_{standard}$).
VSMOW, Vienna Standard Mean Ocean Water.

by Keppler *et al.* (2007). Incorporation of the deuterium label of the ester group in pectin and modified PGAs was also determined using CH_3I. For both quantification and measurement of label, gas chromatography-mass spectrometry (GC/MS) was employed. The GC oven was equipped with a PoraPLOT Q column (12.5 m × 0.25 mm × 8 μm) and programmed to hold at 80°C for 1 min and then ramp at 10°C min^{-1} to 160°C. The injector port was maintained at 250°C and the sample (50 μl) was injected split at a ratio of 100 : 1. The mass spectrometer was operated in the selected ion monitoring (SIM) mode measuring ion currents at *m/z* 127, 142 and 145. CH_3I was quantified by direct comparison of sample peak areas for the sum of ion currents *m/z* 142 and 145 with a calibration curve obtained with authentic standard. Percentage label was calculated as follows: (integral of ion current at *m/z* 145/sum of integrals of ion currents at *m/z* 142 and 145) × 100.

Methoxyl content and degree of labelling for esterified polygalacturonic acids and pectin are presented in Table 1.

Temperature and illumination experiments

Lypholized milled samples (*c.* 200 mg) in glass vials (fused quartz (Suprasil), 40 ml) sealed with caps containing a PTFE-lined silicone septa were heated for 24 h at 40, 60, and 80°C or illuminated with a 250 W Osram Vitalux lamp (UVA 320–400 nm, UV-B 280–320 nm). For more details on the characteristics of the lamp, we refer readers to Vigano *et al.* (2008). All experiments were performed in laboratory ambient air, and thus the initial CH_4 concentration in the vial was approx. 1800–2000 ppb with δ^2H values *c.* –90‰. δ^2H of CH_4 was measured by GC-IRMS at the end of each experiment. For blank measurements, empty vials (containing only laboratory ambient air) were prepared at the same time as the samples and treated under identical conditions.

For the light experiments, vials were placed approx. 35 cm below the lamp, and temperature measured during experiments was in the range between 30 and 38°C. The total unweighted UV-B radiation was *c.* 3.7 W m^{-2} and UV-A radiation was *c.* 42 W m^{-2}. The UV content (UV-A and UV-B separately) was determined with a Waldmann UV meter (Waldmann, Schwenningen, Germany). The relative spectral distribution measurements and the calibration of the Waldmann device were performed with a calibrated standard UV-visible spectro-radiometer (model 752, Optronic Laboratories Inc, Orlando, FL, USA).

Isotope ratio monitoring mass spectrometry for determination of δ^2H values on CH_4

Gas samples were transferred from the vials to an evacuated 40 cm^3 sample loop. CH_4 was trapped on Hayesep D, separated by gas chromatography from interfering compounds and transferred via an open split to the isotope ratio mass spectrometer (ThermoFinnigan Delta^{plus} XL, Thermo Electron Corporation, Bremen, Germany). Concentration (reproducibility ± 20 ppb at ambient concentration) and δ^2H values were determined using a methane standard with known concentration and isotopic composition as internal reference, and a measurement of the inlet pressure in the sample loop. Values of δ^2H (‰) relative to that for Vienna Standard Mean Ocean Water (VSMOW) are defined by the equation δ^2H (‰) = ($^2H/^1H_{sample}/^2H/^1H_{standard}$) – 1. Throughout the paper we use the form $\delta D(CH_4)$ values instead of $\delta^2H_{methane}$ values.

A GC-FID instrument for grab sample analysis (reproducibility ± 10 ppb) was additionally used for verification of the IRMS concentration measurements.

Results and Discussion

In a first approach, we employed pectin which had a low degree (0.05%) of trideuterated methyl groups in the methoxyl moieties (Table 1) for heating experiments at 80°C. The emission rate was measured to be *c.* 2.5 ng g^{-1} DW h^{-1} and the δ^2H values of CH_4 ($\delta D(CH_4)$ values) changed from *c.* –83‰ to 1‰ within 14 h (Table 2). Calculation of the change in δ^2H values, together with the change in CH_4 concentration in the vials, revealed that at least 80% of the

Table 2 Methane emission rates and methane δD values under various detailed experimental conditions

Component	Temperature (°C)	Lamp (Vitalux)	Duration (h)	Blank (ppb)	δD(CH_4) blank (‰)	End (ppb)	δD(CH_4) end (‰)	δD(CH_4) theoretical* (‰)	Emission rate (ng g^{-1} DW h^{-1})
Pectin untreated	80	–	14	1937	–83	2181	–107		3.7
Pectin label 0.05%	80		14	1937	–83	2074	1	18	2.5
PGA untreated	40	–	24	1937	–86	1962	–85		0.2
PGA methyl-esterified 0% label	40	–	24	1937	–86	1981	–87	–	0.3
PGA methyl-esterified 6% label	40	–	24	1937	–86	2029	1 860	2 740	0.7
PGA methyl-esterified 23% label	40	–	24	1937	–86	n.d.	7 310	–	n.d.
PGA untreated	60	–	24	1896	–83	1983	–89		0.7
PGA methyl-esterified 0% label	60	–	24	1896	–83	2012	–89		0.9
PGA methyl-esterified 6% label	60	–	24	1896	–83	2139	12 800	27 500	2.0
PGA methyl-esterified 23% label	60	–	24	1896	–83	2051	54 600	105 000	1.2
PGA untreated	80	–	24	1877	–86	1960	–85		0.6
PGA methyl-esterified 0% label	80	–	24	1877	–86	2225	–124		2.2
PGA methyl-esterified 6% label	80	–	24	1877	–86	2075	20 000	29 400	1.3
PGA methyl-esterified 23% label	80	–	24	1877	–86	2365	126 000	284 000	3.1
PGA untreated	30–38	+	2	1918	–78	n.d.	–84		n.d.
PGA methyl-esterified 0% label	30–38	+	2	1918	–78	2048	–92		9.2
PGA methyl-esterified 6% label	30–38	+	2	1918	–78	2104	7 800	27 600	13.8
PGA methyl-esterified 23% label	30–38	+	2	1918	–78	n.d.	57 500		n.d.
PGA untreated	30–38	+	7	1937	–65	2067	–66	–	2.6
PGA methyl-esterified 0% label	30–38	+	7	1937	–65	2893	–127	–	18
PGA methyl-esterified 6% label	30–38	+	7	1937	–65	3510	39 500	138 000	16
PGA methyl-esterified 23% label	30–38	+	7	1937	–65	3397	163 000	600 000	15
PGA untreated	30–38	+	14	1943	–101	2196	–102	–	2.7
PGA methyl-esterified 0% label	30–38	+	14	1943	–101	3755	–189	–	19
PGA methyl-esterified 6% label	30–38	+	14	1943	–101	3574	59 000	142 000	33
PGA methyl-esterified 23% label	30–38	+	14	1943	–101	4710	233 000	825 000	58

*Theoretical value was calculated by the assumption that the increase of the mixing ratio in the vial (end – blank) comes from methane that is entirely derived from labelled methoxyl groups (see theoretical methane values in Table 1).
Mixing ratios of CH_4 measurements are means of two independent analytical methods (SD ± 23 ppb at ambient concentration).

emitted CH_4 must have been derived from the methoxyl groups of pectin. These first results provided strong evidence of the involvement of pectin methoxyl groups in CH_4 formation. However, because of the complexity of the labelling of the methyl groups in the modified pectin, we decided that it would be more appropriate to utilize another model compound. Therefore, as pectin is a polysaccharide composed primarily of partially esterified α-(1-4)-linked galacturonic acid units, we employed polygalacturonic acid for further experiments. Using polygalacturonic acid as the model compound has a couple of major advantages over pectin itself. Firstly, since it contains no methoxyl groups, it could be used as the control compound to determine if CH_4 formation can also occur from the free acid; and secondly, as it is easily methyl-esterified

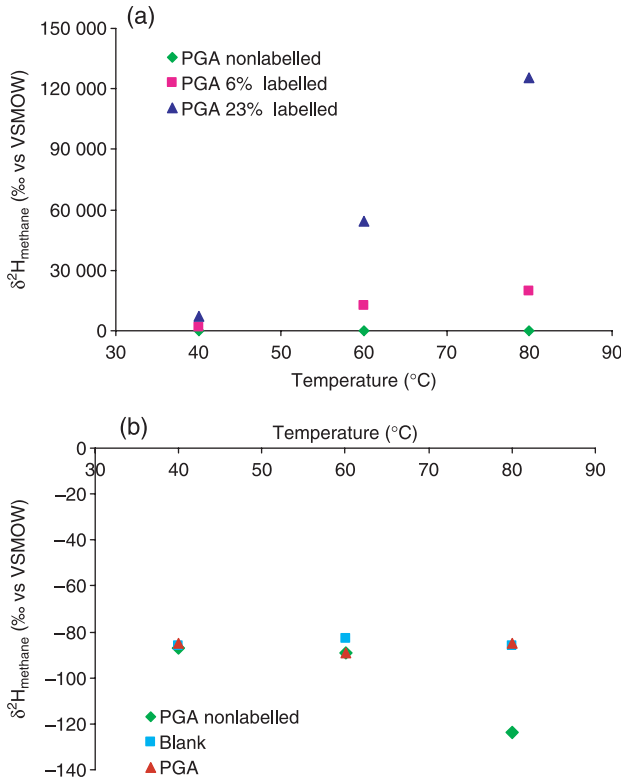

Fig. 2 Heating experiments with polygalacturonic acid (PGA) and methyl-esterified PGAs. (a) $\delta^2H_{methane}$ values of methyl-esterified PGAs; (b) $\delta^2H_{methane}$ values of blank, PGA and nonlabelled methyl-esterified PGA. VSMOW, Vienna Standard Mean Ocean Water.

with methanol, methyl ester derivatives with varying amounts of trideuterium-labelled methyl groups could be conveniently prepared.

Polygalacturonic acids derivatized with anhydrous methanolic H_2SO_4 containing either 0, 5 or 20% tetradeuterated methanol (v/v) were found to contain, respectively, 0, 6.1 and 22.6% trideuterated methyl groups in their methoxyl moieties. From this point forward, these methylated PGAs will be referred to as unlabelled, PGA6 and PGA23. Results from experiments where labelled and untreated PGA samples were incubated at temperatures between 40 and 80°C are shown in Fig. 2. As was expected, high $\delta D(CH_4)$ values were measured for the labelled PGA samples (up to 1 400 000‰, see Table 1) and thus it was essential that ambient laboratory air (*c.* 1900 ppb with $\delta D(CH_4)$ values in the range of −90‰) was used for headspace in the reaction vials so as to avoid massive contamination and memory effects in the analytical system. Headspace from both PGA6 and PGA23 showed a continuous increase in the $\delta D(CH_4)$ values with increasing temperature (Fig. 2a), whereas with unlabelled PGA a slight decrease in $\delta D(CH_4)$ values with increasing temperature (Fig. 2b) was noted. Relative to the labelled samples, the $\delta D(CH_4)$ values of the unlabelled PGA sample only differed marginally from that of laboratory air. The strong increase in the $\delta D(CH_4)$ values for labelled PGA samples is accompanied by an increase in emission rates at higher temperatures. Emission rates for the esterified PGAs were found to range between 0.3 and 0.7 ng g^{-1} DW h^{-1} at 40°C, and between 1.3 and 3.1 ng g^{-1} DW h^{-1} at 80°C, rates in a similar range to that recently reported for apple pectin (Keppler *et al.*, 2006). Formation of CH_4 from untreated PGA was much lower, *c.* 0.2 and 0.7 ng g^{-1} DW h^{-1} at 40 and 80°C, respectively. The calculated ratio of label between PGA23 and PGA6 samples is 3.7 (22.6/6.1), and this ratio was generally reflected in the calculated ratios of the $\delta D(CH_4)$ values of CH_4 formed during the incubation periods. Based on the $\delta D(CH_4)$ values and the increase in headspace CH_4 concentration in the vials during the incubation period, it is possible to calculate the percentage of CH_4 arising directly from the methyl moiety of the methoxyl groups. This calculation shows that, on average, about two-thirds of CH_4 is formed from methoxyl groups, with a range of 48–68% observed (estimated error ± 25%). One possible explanation for this observation is that the methyl moiety is not transferred intact during CH_4 formation. Using GC/MS, with the instrument employed in the selected ion monitoring mode, headspace from PGA23 which had been incubated at 80°C was shown to have peaks at both *m/z* 16 and 19 at the expected retention time of an authentic CH_4 standard. The presence of the peak at *m/z* 19, which was absent in CH_4 formed from incubation of an unlabelled PGA sample, indicated the presence of CH_4 containing three deuterium atoms, an observation which shows that the methyl group from methylated PGA was transferred intact during the heating process. An alternative explanation could be the release of CH_4 as a result of desorption processes, as recently suggested by Kirschbaum *et al.* (2007). As reported earlier, untreated PGA, containing no methoxyl groups, showed small emissions of CH_4 during heating experiments (*c.* 0.2 and 0.7 ng g^{-1} DW h^{-1} at 40 and 80°C, respectively). Since there was no measurable, or only marginal, change in isotope values of the CH_4 observed during these experiments, this indicates that the released CH_4 from the untreated PGA must have a similar value to CH_4 of the background ambient air, and thus it is considered likely that this fraction is derived from desorption processes during heating of the investigated organic material. If we assume that this process is occurring similarly in all conducted experiments using PGA (untreated and methyl-esterified), then we can recalculate the percentage of CH_4 arising directly from the methyl moiety of the methoxyl groups by correcting for CH_4 release of the untreated PGA. In this case, our calculations show values in the range 63–126% (mean value 94 ± 25%), suggesting that methyl-esterified groups can fully explain the *in situ* formation of methane during heating experiments from pectin.

In addition to heating, natural sunlight has been shown to have an even more pronounced effect on CH_4 emissions from pectin (Keppler *et al.*, 2006). Moreover, with the recent

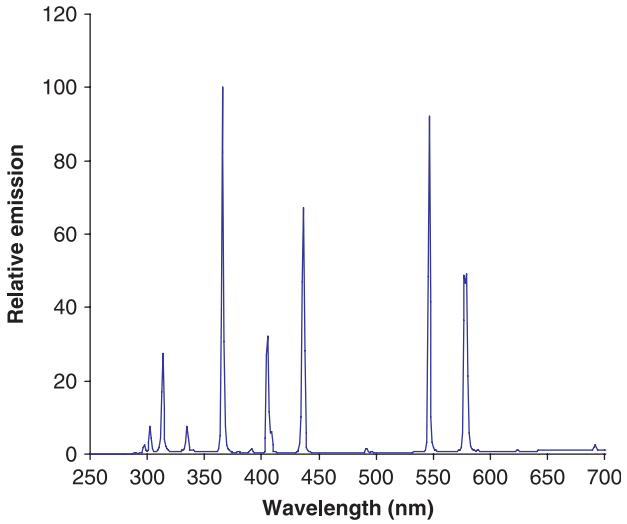

Fig. 3 Spectral distribution of the Osram Vitalux lamp.

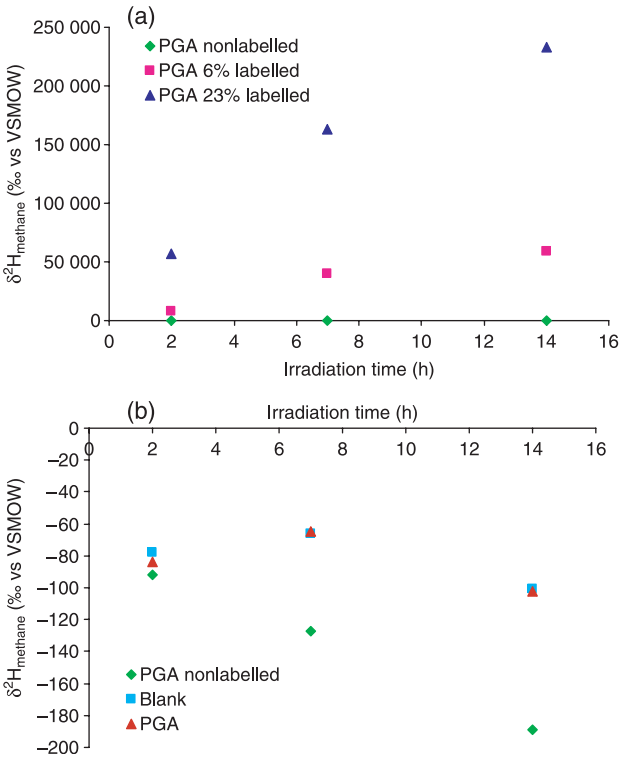

Fig. 4 Irradiation experiments with polygalacturonic acid (PGA) and methyl-esterified PGAs. (a) $\delta^2H_{methane}$ values of methyl-esterified PGAs; (b) $\delta^2H_{methane}$ values of blank, PGA and nonlabelled methyl-esterified PGA. VSMOW, Vienna Standard Mean Ocean Water.

studies of A. R. McLeod (pers. comm.) and Vigano *et al.* (2008), it has now become more evident that UV light plays an important role in the production of CH_4 from plant matter, and for more detailed information about this we refer readers to their work. Thus in a second set of experiments we decided to investigate the influence of UV radiation on isotopically labelled PGA samples. We used a 250 W Osram 'Vitalux' lamp that produces UV-A (320–400 nm), UV-B (280–320 nm) and barely detectable traces of UV-C (< 280 nm) with a spectral distribution shown in Fig. 3 (for more details see Vigano *et al.*, 2008). For our experiments we used a total unweighted UV-B irradiance of *c.* 3.7 W m^{-2}, which is similar to typical UV-B irradiances found in the tropics. Typical ambient (nonweighted) summer UV-B irradiances near the Earth's surface range from 2 W m^{-2} at mid-latitudes to 4 W m^{-2} in the tropics (Bernhard *et al.*, 1997).

This study aimed to demonstrate mechanisms and the molecular source for aerobic production of CH_4 from pectin and the potential role of UV radiation. Realistic environmental UV exposure requires careful attention to spectral distribution and the spectral weighting of experimental lamps (Björn & Teramura, 1993), so further studies are required to ensure accurate simulation of ambient UV exposures and the extent and quantification of these processes in natural sunlight. Similar to the heating experiments, CH_4 formed from both PGA6 and PGA23 samples showed an increase in the $\delta D(CH_4)$ values with increasing irradiation time (Fig. 4a), reaching values of up to *c.* 230 000‰ after 14 h whilst the unlabelled PGA sample showed a significant decrease from −101 to −189‰ over the same time period (Fig. 4b). $\delta D(CH_4)$ values of the untreated PGA did not differ significantly from that of laboratory air. Emission rates from untreated PGA were found to range from 2 to 3 ng g^{-1} DW h^{-1}, whilst, in contrast, rates for all methylated PGAs were found to range between 9.2 and 36 ng g^{-1} DW h^{-1}, which is one to two orders of magnitude higher than the rates measured during heating experiments at 40°C. With light, the percentage of CH_4 calculated to be directly derived from methoxyl groups ranged from 28 to 41%, with, on average, one-third found to originate from this source. Correcting these values for the observed methane release from untreated PGA, the percentage of CH_4 calculated to be directly derived from methoxyl groups increases to *c.* 50% (mean value 51 ± 25%); values for all samples being in the range 30–86%. This proportion differs from that observed for the temperature experiments, possibly indicating different pathways involved in CH_4 formation in the two processes. It should be mentioned that the CH_4 fraction not originating directly from the methyl moiety of the methoxyl pool cannot be fully explained by formation from nonesterified PGA, as those emission rates are almost an order of magnitude lower than the rates observed for methoxylated PGAs. Therefore it would appear that esterification of the carboxyl moiety in pectin is also important for the observed secondary CH_4 formation process during UV-light experiments, in that once the methyl group is removed, it increases the possibility of CH_4 formation from other carbon atoms within the PGA structure.

An example of a free radical process leading to formation of CH_4 during photochemical-induced degradation of polysaccharides was recently presented by Sharpatyi (2007). Although

free radical processes are known to generate a cascade of reactions, from a chemical viewpoint CH_4 formation from carbon moieties other than methoxyl of PGA is difficult to envisage. The significance of this pathway of CH_4 for aerobic formation in plant tissue will require further intensive isotope labelling investigations.

Conclusions

Our results provide unambiguous isotope evidence that methoxyl groups of pectin can act as a source of atmospheric CH_4 under aerobic conditions. As previously shown (Keppler *et al.*, 2006), emissions of CH_4 from pectin are strongly dependent on temperature and exposure to light, in particular in the UV range.

For more detailed studies on the light effect, we would refer readers to the study of Vigano *et al.* (2008), in which the role of UV light in the formation of methane from dried and fresh detached leaves is described in considerable detail. Although the mechanism is still unknown, this study is an important first step in gaining more knowledge of potential plant precursor components that will enable delineation of the reaction mechanism, an essential requirement to fully understand the environmental significance of aerobic methane formation.

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