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The localisation of pectin in *Sphagnum* moss leaves and its role in preservation

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ABSTRACT

The localisation of pectin in *Sphagnum* moss leaves and its role in preservation has been investigated. Light microscopy using ruthenium red to detect pectin in whole and sectioned *Sphagnum papillosum* leaves revealed it is abundant in hyaline cell walls, fibrils, papillae, chlorophyllous cell walls and thickenings around hyaline cell pores. Transmission electron microscopy of ultrathin cell walls labelled with poly-L-lysine colloidal gold revealed pectin was distributed throughout the cell wall. The preservative/microbiocidal properties of these pectins are explained by the acid-dissociation properties of galacturonic acid carboxyls and their incorporation in the unique cell arrangement of the *Sphagnum* leaf. Liquid from a salmon fillet absorbed into *S. papillosum* leaves and incubated at room temperature for 22 h had a pH around 4.85, was dominated by *Lactobacillus* sp. and smelled fresh compared to experimental controls. Chlorite-treated *Sphagnum* leaves could have a potential as a food tray pad that absorbs liquid and prevents the growth of spoilage bacteria inside it.

1. Introduction

Earlier experiments have shown that *Sphagnum* moss displays microbiocidal properties under certain conditions (Børsheim, Christensen, & Painter, 2001). When used as a packing material it had the ability to seemingly preserve and slow-down the on-set of putrefaction of whole Zebra fish for a number of days at room temperature and in the presence of oxygen (Børsheim et al., 2001). Historical evidence for the preservative properties of *Sphagnum* moss is also abundant. Sterile *Sphagnum* moss bandages show excellent wound healing properties and their use dates back to the Bronze Age (Varley & Barnett, 1987a, 1987b). In particular they were extensively used in World War I (Riegler, 1989). *Sphagnum* moss is also used in Scandinavia to preserve post-harvest fruit and vegetables, and even freshly caught fish.

Until recently it was thought that a novel uronic acid residue with a reactive carbonyl group, 5-keto-D-mannuronic acid (5-KMA), residing in pectin-like polysaccharides of the plant cell wall was responsible for the observed antimicrobial and preservative effect. Re-analysis though showed 5-KMA does not occur in *Sphagnum papillosum* and probably not in other *Sphagnum* species (Ballance, Borsheim, Inngjerdingen, Paulsen, & Christensen, 2007; Ballance, Kristiansen, Holt, & Christensen, 2008). It appears to be the carboxyl group of galacturonic acid (GalA) in pectin, which accounts for between 10 and 30% of the dry weight of *Sphagnum*, that are the chemical entities responsible for its observed microbiocidal properties (Stalheim, Ballance, Christensen, & Granum, 2009). These carboxyl groups in their initial acid form are microbiocidal in some instances because as a function of pH they release protons into solution in exchange for cations. This property is explained by standard acid dissociation theory i.e. the acid dissociation constant of the galacturonic acid carboxyl.

It is, nevertheless, the unique material properties of *Sphagnum* leaves that also confer these microbiocidal properties of pectin to intact cell and leaf structure The leaves are one cell thick with the majority of volume (c.a. 90%) comprising dead, long, empty, thin-walled cells known as hyaline cells with circular or elliptical pores of between 3 and 22 μm in diameter (Clymo & Hayward, 1982). Liquid enters the hyaline cell of the leaves mostly via mass flow through the pores. Thus a lot of water absorbed by *Sphagnum* leaves is inside the hyaline cells at water potentials less than −10 kPa (Clymo & Hayward, 1982). At higher water potentials more water is held in external capillary spaces between the leaves (and branches) (Hayward & Clymo, 1982). Given the high abundance of

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Table 1
Carbohydrate composition and Klason lignin content of dried *S. papillosum* leaves, chlorite-treated leaves, and chlorite–carbodiimide–sodium borohydride treated leaves. Carbohydrate values are expressed as dry weight % polysaccharide.

Component	Amount (dry weight %)		
	Dried leaves (H^+-form)	Chlorite-treated leaves (H^+-form)	Chlorite–carbodiimide–sodium borohydride treated leaves (Na^+-form)
Rhamnose	3.11	4.43	11.58
Fucose	0.3	0.26	0
Arabinose	0.88	0.85	1.4
Xylose	4.84	6.01	8.43
Mannose	4.39	5.24	5.99
Galactose	5.87	7.22	14.79
Glucose	27.13	35.21	40.26
Galacturonic acid	13.6	17.5	7.14
Klason Lignin	16.5	3	N.D.
Total	76.62	79.72	90.24

GalA in the hyaline cells, coupled with their close proximity to the hydrated internal/external environment, these may participate in acid dissociation, which in a solution in a closed system where the buffering capacity is low enough, can result in a drop of pH (Clymo, 1963; Richter & Dainty, 1989).

It is for these reasons we believe the moss may be antibacterial towards acid sensitive bacterial species, which can pass into the hyaline cells, or are in a liquid media where the quantity of the moss exceeds the buffering capacity of the growth media. Indeed only acid tolerant bacterial species predominantly thrive in the hyaline cells of natural *Sphagnum* (Opelt & Berg, 2004; Raghoebarsing et al., 2005). Together these factors probably go some way to explain the extensive current and past uses of *Sphagnum* moss mostly in the form of the raw plant tissue. It is, however, known that *Sphagnum* treated with chlorite seems to retain all the functional properties described above but with the additional bonus that it is white, sterile, enriched in uronic acids, can be converted into its full acid-form to remove unwanted heavy metal contamination and generate maximum acidity. It is essentially free of aromatics, water repellent waxes and other cell components (Painter & Sorensen, 1978; Richter & Dainty, 1989). All these additional properties may allow the commercial potential of *Sphagnum* moss, or an environmentally friendly synthetic analogue, as an antimicrobial substance to be expanded into new markets additional to those of today which just utilise the dried moss. One of these markets, which have not been extensively exploited, is the area of food hygiene and active packaging. Applications that take advantage of the ability of the hyaline cells or similar synthetic structures to soak-up, hold, inhibit or slow-down growth of acid sensitive bacteria in a liquid medium would seem quite promising and which we consider warrants further investigation. A recent study looked at the possibility of using chlorite-treated *Sphagnum* in the form of a pad placed in a modified atmosphere packed (MAP) food package as a means to increase the shelf-life of a salmon fillet (Iotti, Fava, Ballance, Christensen, & Rustad, 2006). Despite no difference between the experimental control, in terms of orders of magnitude reduction of bacterial colony forming units on the salmon fillets after 30 days storage, there was no adverse affect on salmon fillet texture and a marked reduction in off odour compared to controls (Iotti et al., 2006).

The aims of our current study are therefore to first study the localisation of pectin within the *Sphagnum* leaf. To date this has not been studied in much detail apart from the indication that pectin carboxyls are unequally distributed (Richter & Dainty, 1990). Secondly, we show the acidification properties of chlorite-treated leaves can have an applied purpose i.e. as a food tray, by suppressing the growth of acid sensitive food spoilage bacteria.

2. Materials and methods

2.1. Production of chlorite-treated and subsequently carboxyl-reduced Sphagnum moss

S. papillosum was treated with chlorite as described earlier (Ballance et al., 2007) to remove the majority of waxes and aromatic components (see Klason lignin analysis, Table 1). The carboxyls were then activated with carbodiimide and reduced with sodium borohydride as described previously (Hájek, Ballance, Limpens, Zijlstra, & Verhoeven, 2011). Chlorite-treated moss was also directly reduced with 2% (w/v) sodium borohydride as described earlier (Børsheim et al., 2001). All samples were converted to their acid form prior to experimentation (Børsheim et al., 2001).

2.2. Determination of neutral sugar, uronic acid, moisture content and Klason lignin

Finely milled samples were stored at 4 °C. For neutral sugars equivalents of 5–15 mg were weighed out into a hydrolysis tube. The sample was then incubated in 0.5 ml 12 M H_2SO_4 for 30 min at 35 ± 1 °C followed by addition of 2.5 ml water to make the solution 2 M H_2SO_4 and incubated in a boiling water bath for 1 h. The liberated sugars were then converted into their corresponding alditol acetates and quantified via monosaccharide standards (Englyst, Quigley, & Hudson, 1994) which compensate for destruction of released monosaccharides upon liberation from their parent polysaccharide. Alditol acetates were chromatographed on a DB-23 silica capillary column using flame ionization detection on an Agilent GC. Data were collected and processed using Agilent Chemstation software. Uronic acid was determined by the meta-hydroxydiphenyl assay after its liberation via treatment with sulphuric acid (Ahmed & Labavitch, 1978). All values are expressed as grams polysaccharide per 100 g sample dry weight. 0.89 was used as a universal multiplication factor to convert neutral monosaccharide to polysaccharide and 0.9 to convert uronic acid to polysaccharide. Moisture content was determined in a Sartorius Thermo Control YTC 01 L infra red dryer.

Klason lignin was determined by swelling ca. 0.5 g sample in 12 M H_2SO_4 for 30 min at 35 °C followed by hydrolysis in boiling water for 1 h in 2 M H_2SO_4 after the addition of 74 ml water. The insoluble material was then collected and washed in a pre weighed glass-fritted crucible and dried overnight at 105 °C and re-weighed. The sample was then placed in a muffle furnace and incubated at 500 °C for 1 h, cooled in a desiccator and the crucible and ash weighed. Klason lignin was determined via weight loss

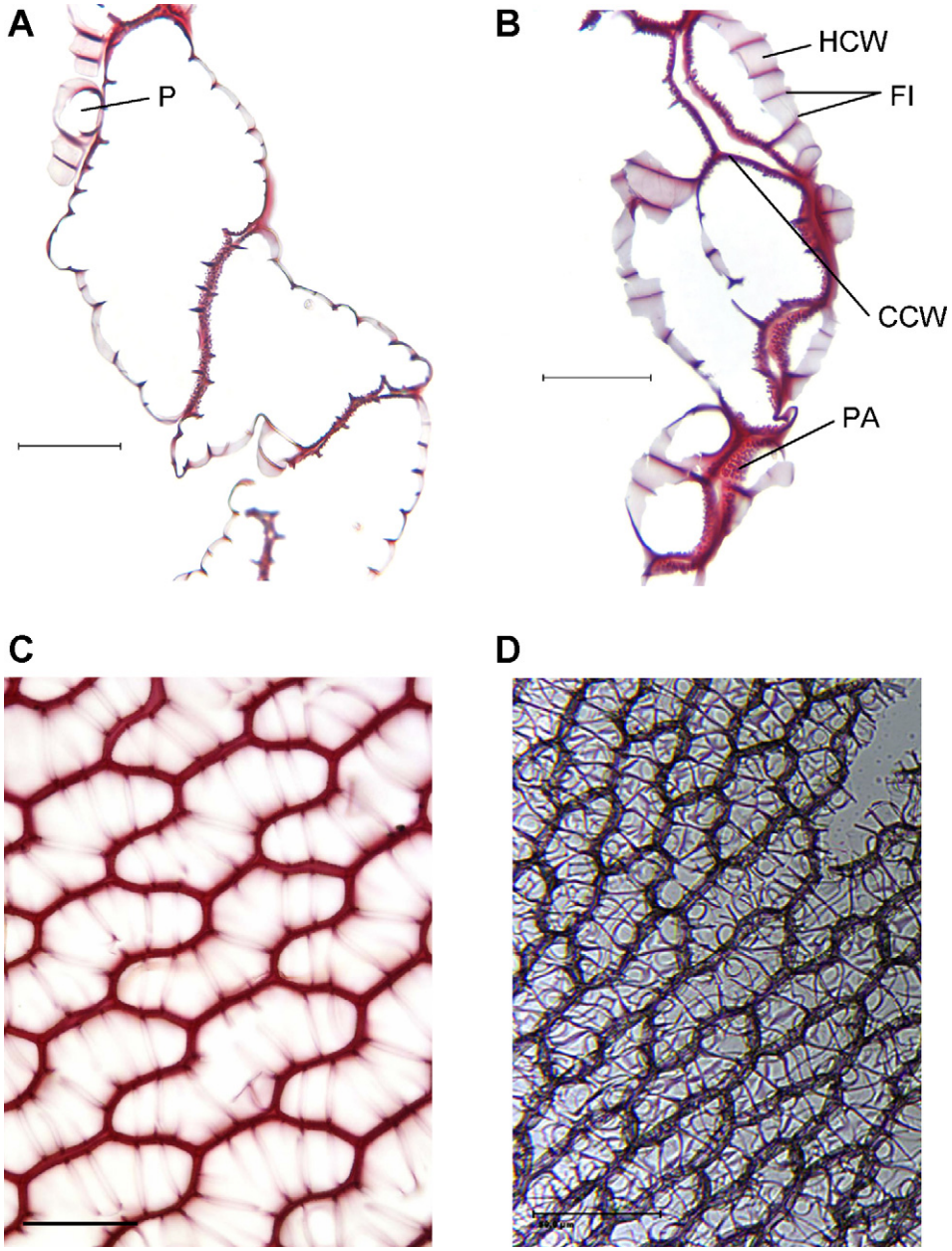

Fig. 1. Thin-section of a dried *S. papillosum* leaf (A) and chlorite-treated leaf (B), whole chlorite-treated *S. papillosum* leaf (C) and whole chlorite–carbodiimide–sodium borohydride treated *S. papillosum* leaf (D) stained with ruthenium red and viewed under the light microscope. **PA** are papillae which are located only on the inner luminal cell walls of the hyaline cells (**HCW**) where they are in contact with the chlorophyllous cell wall (**CCW**). **FI** are fibrils that occur as a spiral structure inside the hyaline cell. **P** is a pore. Scale Bar = 50 μm.

and calculated as described earlier (Theander, Aman, Westerlund, Andersson, & Petersson, 1995).

2.3. Light microscopy of ruthenium red stained wet mounts and thin sections

Dried whole leaves were stained with 0.02% (w/v) ruthenium red in 1% ammonium acetate (ammoniated ruthenium oxychloride) for 10–15 min (Kremer, Pettolino, Bacic, & Drinnan, 2004). The mechanism of staining is electrostatic condensation. These were then washed three times in changes of 0.1 M NaCl, followed by water and finally xylene prior to mounting on a glass slide with Cytoseal. In addition leaves were dehydrated in a graded concentration series of ethanol washes of 50, 70, 90, 96% and absolute (v/v), cleared in xylene and embedded in paraffin wax. Thin sections of 4 μm were cut on a microtome, mounted on a slide and de-paraffinated in xylene, re-hydrated in a graded series of ethanol washes and stained with ruthenium red as above. All preparations were viewed with a Nikon Labophot-2 microscope and images captured with an RT-Color SPOT camera (Diagnostic Instruments Inc, USA).

2.4. Transmission electron microscopy of colloidal gold-poly-L-lysine labelled ultra thin sections

Fresh living *S. papillosum* branches of leaves where picked on 20 September 2007 from a mire on the outskirts of Stjørdal, N. Trøndelag, Norway, and immediately fixed *in situ* in a mixture of

2.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Dried fresh *S. papillosum* leaves and chlorite-treated leaves and carboxyl-reduced chlorite treated leaves were fixed in an identical way. All samples were fixed for a least 1 h at room temperature followed by at least overnight at 4 °C. Prior to dehydration and embedding in LR White resin, all fixed samples were thoroughly washed in 0.1 M phosphate buffer, pH 7.2 in 3 repeated 2 h cycles and once overnight. The samples were dehydrated in a graded concentration series of ethanol, 50% (v/v) 15 min, 70% (v/v) 2 × 15 min, and 73% (v/v) 2 × 15 min. The dehydrated leaves were then incubated at 4 °C for 72 h in a 2:1 mixture of Pelco Medium Grade LR White resin: 73% (v/v) ethanol followed by 2 × 100% LR White resin for 24 h and 2 × 100% LR White resin for 2 h on a rotator. The samples were finally placed in gelatine capsules in fresh LR-White resin and incubated at 60 °C for 24 h to polymerise/cure the resin.

Ultrathin sections of 90 nm thickness were cut from 4 replicate embedded samples using a Leica EM UC6 ultramicrotome. These were mounted on 200 mesh nickel grids coated with formvar to support the sections. The grids were then immersed in 30 mM acetate buffer, pH 5.5 (*ionic strength* = 0.03 M) for 15 min followed by 1 h in a bath of poly-L-lysine-gold 10 nm colloidal gold complex (BBI international) diluted 1:60 in acetate buffer. The mechanism of labelling is complex coacervation to form a pectin-poly-L-lysine-gold complex. Following this treatment grids were washed in 20 changes of acetate buffer and then in 10 changes of water. After labelling all sections were contrast stained with 4% uranyl acetate in 50% (v/v) ethanol followed by 1% lead citrate in 0.1 M sodium hydroxide. All sections were examined with a Jeol JEM-1011 transmission electron microscope (TEM) operating at 80 kV.

Controls to assess labelling specificity were: (1) incubation of sections with poly-L-lysine colloidal gold pre-incubated and thus inactivated with 1 mg/ml pectin (sphagnan) previously extracted from *S. papillosium* (Ballance et al., 2007). (2) Pre-incubation of sections with 1 mg/ml poly-L-lysine (Sigma, P-1524 – the same preparation as attached to the colloidal gold).

For examination of general morphology of ultra-thin sectioned leaves all preparations with the exception of fresh leaves were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, embedded in expoxy-resin (LX112), sectioned, stained with uranyl acetate and lead citrate as described above and viewed under TEM (Jeol JEM-1011).

2.5. Experiments with chlorite-treated Sphagnum with dirty dishwater and fish fillet exudate

Six dinner plates soiled after a meal of potatoes, meat and gravy were selected at random from a commercial kitchen. After approximately 18 h storage at room temperature the dirty plates were cleaned (without detergent) in warm (ca. 40–45 °C) tap water. The dirty dishwater was left to cool for about 1 h. To 50 ml plastic syringes (3 replicates for each treatment) containing either 1 g dried chlorite-treated *Sphagnum* or 1 g viscose textile (dishcloth) or nothing, 20 ml of the dirty dishwater was added. The syringes were left upright and shaken on a shaking table for 18 h without the plunger – open to the atmosphere and at room temperature. Following incubation the smell of the contents of the different treatments in the syringe was recorded by a method described previously (Iotti et al., 2006). The plunger was then used to press out liquid absorbed and surrounding the chlorite-treated moss and that surrounding the viscose. To the liquid collected after incubation the pH was immediately recorded and 100 μl of the liquid was immediately diluted in 900 μl 0.9% (w/v) NaCl (saline) and plated out on Difco plate count agar together with serial 10-fold dilutions in saline. The plates were incubated at 22 °C for 18 h and colony forming units (CFU) counted. This experimental cycle was repeated another four times over the course of a week using newly prepared dishwater each time. The initial dishwater inoculum had a viable bacterial CFU $ml^{-1} < 10^2$.

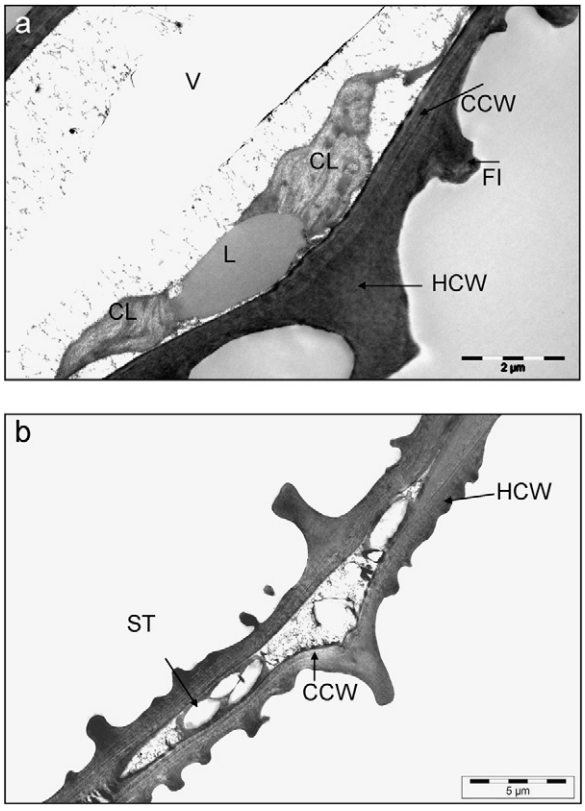

Fig. 2. Ultra-thin section of expoxy-resin embedded dried *S. papillosum* chlorphylous cell with fragments of hyaline cell, contrast stained with uranyl acetate and lead citrate, and examined by TEM. **CL** is a chloroplast, **L** is a lipid body, **ST** are starch granules and **V** is a vacuole all of a chlorphylous cell. The annotation of other features is as Fig. 1. Scale bar = 2 and 5 μm respectively for A and B.

A similar experimental set-up was applied to study the effect of salmon exudate on the pH and microbiological growth inside/surrounding the hyaline cells. Four 140 g frozen salmon fillets in individual plastic packs were purchased from a local shop and defrosted for 4.5 h at room temperature. The accumulated liquid inside the packs was collected and pooled. The top-layer lipid phase was discarded. To 20 ml plastic syringes (3 replicates for each treatment) containing either 0.5 g dried chlorite-treated *Sphagnum* or 0.5 g 1 cm^2 pieces of standard food tray pads made from cellulose or nothing, 8 ml of the fish exudate was added. Incubation, sampling and measurements were as described above. The experiment was stopped after one incubation cycle.

3. Results and discussion

The GalA content of dried *S. papillosum* leaves and chlorite-treated leaves (Table 1) was at the lower end of the range found for other *Sphagnum* species (Clymo, 1963; Craigie & Maass, 1966; Hájek et al., 2011; Spearing, 1972). Forty percent of these uronic acids also remained in the chlorite-leaves subjected to a carbodiimide–borohydride carboxyl reduction (Table 1). Incomplete reduction of carboxyls by this method, even for polysaccharides in solution, is somewhat typical (Stenutz, 2010). Nevertheless whole or thin sections of leaves treated with carbodiimide–borohydride did not avidly bind and stain with ruthenium red (Fig. 1d). Cell walls of dried *S. papillosum* leaves, chlorite-treated leaves (Fig. 1a–c) and chlorite treated leaves incubated in sodium borohydride (result not shown) all stained positive

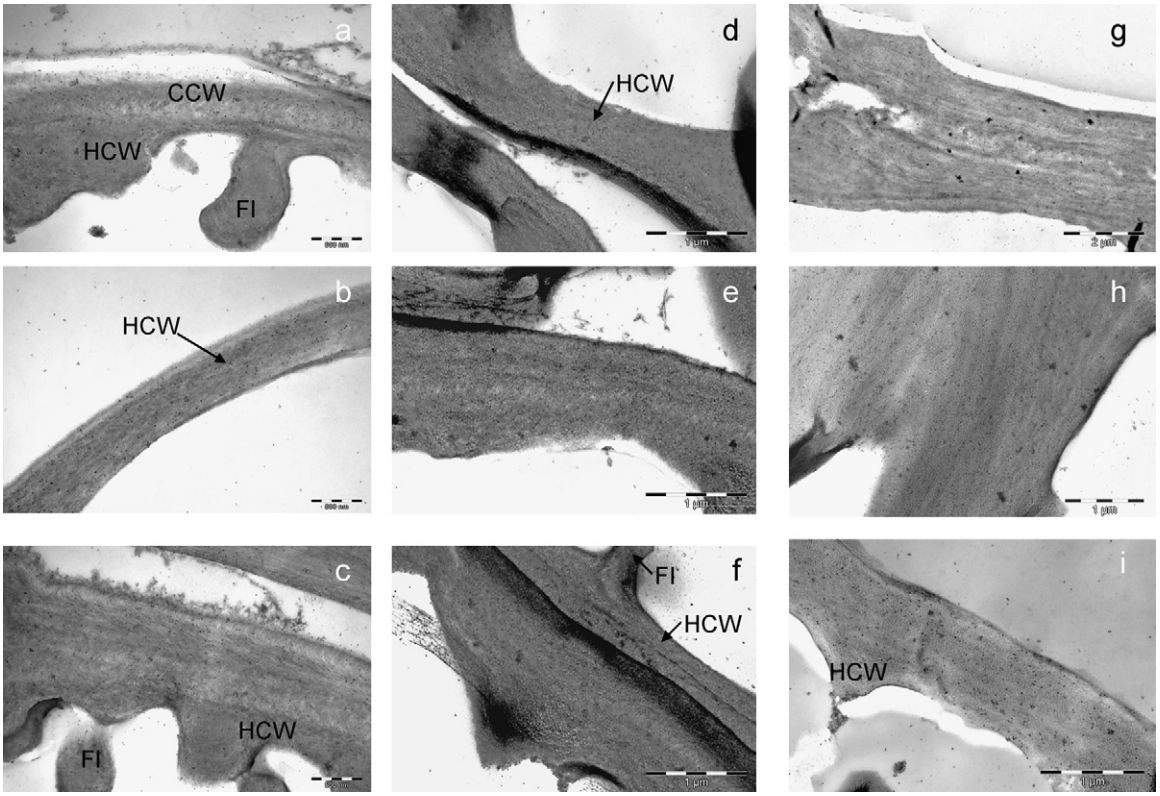

Fig. 3. Transmission electron microscopy of ultra-thin sections of fresh LR White embedded *S. papillosum* leaf (A–C), treated with chlorite (D–F), treated with chlorite–carbodiimide–sodium borohydride (G–I). All preparations have been exposed and labelled with poly-L-lysine-colloidal gold which can be seen as small black dots (spheres) on the chlorophylous and hyaline cell walls. The annotation of identifiable features is as for Figs. 1 and 2. Scale bars: A–C = 500 nm, D–F, H–I = 1 μm, G = 2 μm.

with ruthenium red. It is thought a stoichiometric relationship of n-2 ruthenium red molecules per anhydrogalacturonide unit occurs in pectin (Sterling, 1970). Due to its size and hexavalent cationic nature, condensation with carboxyls by bridging cannot be ruled out (Luft, 1971). Our observations with ruthenium red staining, before and after carbodiimide–borohydride treatment (Table 1 and Fig. 1), suggests a large proportion of the total carboxyl groups in *S. papillosum* leaves participate in ion-exchange/acid dissociation. This fits with the general observation of a good correlation between the uronic acid content and cation exchange ability at pH < 7 (Clymo, 1963).

Closer inspection of thin-sections of chlorite treated leaves stained positively with ruthenium red showed GalA appeared to be rich in cell wall structures (Fig. 1). These include the fibrils of hyaline cells, papillae lining the luminal part of the hyaline cell walls in contact with the chlorophyllous cell wall (Fig. 1a and b) as well as chlorophyllous cell walls themselves and around the cell wall thickenings of hyaline cell pores. However, these structures are also thicker than the rest of the cell wall. At the spatial resolution of the light microscope it does not necessary mean then that the 3D-density of carboxyl groups in *Sphagnum* cell wall regions differ.

Two monoclonal antibody probes JIM5 and JIM7, specific for unesterified and methylesterified homogalacturonan respectively, did not bind to any epitopes at the ultrastructural level in ultra-thin sections of *S. novo-zealandicum* examined with TEM (Kremer et al., 2004). This is consistent with the finding that pectin in *Sphagnum* is similar in form to rhamnogalacturonan I (Ballance et al., 2007) which is non-reactive with these antibodies (Jauneau, Roy, Reis, & Vian, 1998). Instead we opted to use poly-L-lysine conjugated to colloidal gold. This generic probe has been successfully used to study the ultra-structural localisation of pectin in apple and kiwi fruits (Roy et al., 1994; Sutherland, Hallett, Redgwell, Benhamou, & MacRae, 1999). With help of Fig. 2 for orientation, Fig. 3 shows that pectin in fresh, dried and chlorite-treated *Sphagnum* leaves are more or less spread throughout the hyaline and chlorophylous cell wall. Interestingly the same preparation of whole chlorite–carbodiimide–borohydride treated leaves, which did not stain with ruthenium red, had a labelling intensity and specificity similar to the other *S. papillosum* leaf preparations when ultra-thin sections were examined under TEM after interaction with poly-L-lysine colloidal gold (Fig. 3). This may mean uronic acids located internally in the cell wall were less prone to carboxyl reduction than on the cell wall surface, or more likely, the poly-L-lysine molecule with a molecular weight >300,000 was easily capable of bridging neighbouring pectin chains of remaining unreduced carboxyl groups through multiple binding sites to form a pectin-poly-L-lysine polyelectrolyte complex.

No labelling of *Sphagnum* cell constituents with poly-L-lysine colloidal gold occurred in negative control samples when poly-L-lysine-colloidal gold was pre-incubated with excess pectin extracted from *S. papillosium,* prior to exposure to ultra-thin cell wall sections (Ballance et al., 2007). Similarily no labelling was observed when ultra-thin cell wall sections were pre-incubated with excess poly-L-lysine only prior to exposure to poly-L-lysine-colloidal gold. Non-specific background staining of the formvar film on which the ultrathin sample is mounted was negligible (Fig. 3). These observations confirmed the specificity of binding of poly-L-lysine to GalA as noted in similar studies of apple pectin (Sutherland et al., 1999). Although previous studies have labelled pectins with poly-L-lysine at pH 2.6 we found much better and more specific labelling at pH 4.5–5.5. Previous studies have shown this slightly higher pH range is optimal for the formation of gelatin-*Sphagnum* pectin polyelectrolyte complexes (Ballance et al., 2008) and seems to be the case for poly-L-lysine-*Sphagnum* pectin complexes too.

Table 2
pH ± SEM ($n = 3$), smell and bacterial CFU ml^{-1} ± SEM ($n = 3$) in dirty dishwater incubated in four repeated cycles of 22–24 h at room temperature in chlorite treated *Sphagnum* leaves, in a solution inside and outside a viscose cloth, and the initial inoculum on its own. * pH in innoculum: ($n = 1$). ** Remaining replicate plates – too many colonies to count.

Treatment		Day 0	Day 1	Day 2	Day 3	Day 4
Start inoculum	CFU ml^{-1}	<10	<10	<10	<10	<100
	pH*	7.33	7.34	7.33	7.21	6.56
Chlorite-treated	CFU ml^{-1}		<10 ± 0.00	<10 ± 0.00	<10 ± 0.00	<10 ± 0.00
	pH		3.23 ± 0.15	3.36 ± 0.10	3.65 ± 0.10	3.57 ± 0.10
	Smell	Mild	Mild	Mild	Mild	Mild
Viscose	CFU ml^{-1}		$5 \times 10^6 \pm 2 \times 10^6$	$2 \times 10^9 \pm 0.5 \times 10^6$	$5 \times 10^6 \pm 1 \times 10^6$	**$>2.76 \times 10^{11}$
	pH		6.63 ± 0.14	6.97 ± 0.06	6.70 ± 0.06	6.65 ± 0.12
	Smell	Mild	Sour/rotten	Sour	Sour	Sour

Table 3
pH ± SEM ($n = 3$), smell, colour and bacterial CFU ml^{-1} ± SEM ($n = 3$) in salmon exudate incubated for 22 h at room temperature in chlorite-treated *Sphagnum*, leaves, inside the structure of a standard food tray pad, and exudate on its own (control). ** Remaining replicate plates – too many colonies to count. N.D. = not determined.

Treatment	pH	CFU ml^{-1}	Smell	Colour of exudate	Predominant bacteria type
Chlorite-treated	4.85 ± 0.07	$6.7 \times 10^9 \pm 1.6 \times 10^9$	Fresh salmon	White	*Lactobacillus* sp.
Standard pad	5.55 ± 0.04	**$>3 \times 10^{10}$	Rotten	Pale yellow	N.D.
Fish exudate only	6.00 ± 0.02	**$>3 \times 10^{10}$	Weak rotten	Salmon pink	N.D.

At the macroscale the apparent relatively even distribution of pectin throughout both secondary hyaline and chlorophylous cell walls shows that, unlike polyphenolic residues which just appear to coat the cell wall surface (Tsuneda, Thormann, & Currah, 2001), these polysaccharides are essential for cell wall integrity. This notion is supported by the observation that leaf structure remains intact after chlorite-treatment, which removes the majority of the lignin-like, polyphenolic residues (Table 1, Richter & Dainty, 1989). On the other hand if one incubates chlorite-treated leaves in their acid form in hot water the pectin is released into solution and the hyaline cell walls start to loose their structural integrity (Ballance et al., 2007). It therefore seems that pectin may have a direct or indirect structural function in the cell wall. Pectin in *Sphagnum*, via its high concentration of carboxyls, may also play an important role in water sorption (Clymo & Hayward, 1982). It is interesting to observe that papillae (Fig. 1b) strongly stain with ruthenium red and therefore appear rich in pectin. At present the function of papillae in mosses is largely unknown (Câmara & Kellogg, 2010) although they only occur in two *Sphagnum* species: *S. affine* and *S. papillosum*.

An experiment with a repeated exposure of *Sphagnum* or viscose to a dirty dishwater bacterial inoculum demonstrates the role of acid dissociation from galcturonic acid in acidification (Table 2). Where pH was below pH 3.7 in the presence of chlorite-treated *Sphagnum* bacterial growth was suppressed <10 CFU ml^{-1}. In contrast, and when a viscose cloth was placed in the inoculum, pH was between 6.5 and 7 after 24 h incubation (Table 2). During this time period bacteria multiplied rapidly from <10 to >10^6 CFU ml^{-1} (Table 2). These observations support the hypothesis proposed in the introduction and outlined in a previous study (Stalheim et al., 2009) that both pectin in *Sphagnum* leaves, and its unique material properties in absorption of liquid, can have a major impact on microbiology by lowering the pH of a bacterial growth medium.

In experiments with 22 h incubation of salmon exudate absorbed into/surrounding the leaves *Lactobacillus* sp. multiplied rapidly and the final pH was 4.85 (Table 3). Where a standard cellulose food tray pad or just fish exudate was incubated instead of *Sphagnum* the pH was 5.55 and 6 respectively (Table 3). Bacterial growth was at a similar magnitude (Table 3). It is well known that *Lactobacillus* thrives in weakly acidic conditions, which are inhibitory to many fish spoilage and common human pathogens (Francoise, 2010). Certain strains of *Lactobacillus* can also actively suppress the growth of the undesirable food poisoning bacteria such as *Listeria monocytogenes*, which can also grow in weakly acidic conditions, via the production of anti-listerial peptides (Katla et al., 2001). In addition *Lactobacillus* often produces few unpleasant odours compared to common fish spoilage bacteria such as *Shewanella* (Francoise, 2010) and this was confirmed in the present study (Table 3).

Overall then a food tray pad made from chlorite-treated *Sphagnum* may have an advantage over conventional paper pads in that growth of food spoilage bacteria are suppressed inside the *Sphagnum* based pad. If such a technology could also be combined with inoculation of the pad or fish fillet with a *Lactobacillus* protective culture prior to packaging then the unwanted side-effect of acidification in the hyaline cells – the selection for and growth of *Salmonella* or *Listeria monocytogenes* could be actively prevented. At least for fish products the application of protective cultures as a method of biopreservation is still in its infancy (Francoise, 2010). However, an active food packaging material with similar properties to *Sphagnum* moss that contains the beneficial preservative properties, such as the production of acid and absorption of liquid, without markedly altering the sensory attributes of the preserved food is of both potential commercial and consumer interest. Further research is required if this approach should become a viable reality.

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