Aluminium effects on mechanical properties of cell wall analogues

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Aluminium (Al) toxicity adversely impacts plant productivity in acid soils by restricting root growth and although several mechanisms are involved the physiological basis of decreased root elongation remains unclear. Understanding the primary mechanisms of Al rhizotoxicity is hindered due to the rapid effects of soluble Al on root growth and the close proximity of many cellular components within the cell wall, plasma membrane, cytosol and nucleus with which Al may react. To overcome some of these difficulties, we report on a novel method for investigating Al interactions with *Komagataeibacter xylinus* bacterial cellulose (BC)-pectin composites as cell wall analogues. The growth of *K. xylinus* in the presence of various plant cell wall polysaccharides, such as pectin, has provided a unique in vitro model system with which to investigate the interactions of Al with plant cell wall polysaccharides. The BC-pectin composites reacted in a similar way with Al as do plant cell walls, providing insights into the effects of Al on the mechanical properties of the BC-pectin composites as cell wall analogues. Our findings indicated that there were no significant effects of Al (4–160 μM) on the tensile stress, tensile strain or Young's modulus of the composites. This finding was consistent with cellulose, not pectin, being the major load bearing component in BC-pectin composites, as is also the case in plant cell walls.

Abbreviations – BC, bacterial cellulose; HS, Hestrin and Schram; KHP, potassium hydrogen phthalate.

Introduction

Komagataeibacter xylinus is a cellulose synthesizing bacterium that is an established archetype for cellulose biogenesis (Brett 2000). The growth of *K. xylinus* in the presence of various plant cell wall polysaccharides, such as pectin and xyloglucan, results in the formation of polysaccharide composites that mimic plant cell walls (Chanliaud and Gidley 1999). This unique *in vitro* model system has been used to investigate the interactions of these plant cell wall polysaccharides and their resultant physical properties (Astley et al. 2001, Chanliaud et al. 2002, Chanliaud and Gidley 1999, Tokoh et al. 2002, Touzel et al. 2003, Whitney et al. 1995). The composites, in their natural hydrated state, typically more than 90% water, mimic the hydration state of primary plant cell walls. Analogous to the deposition of plant cell walls bacterial cellulose microfibrils are extruded into the extracellular medium containing pre-formed pectic gel matrix, with Ca as the reticulating cation. The mechanical properties of BC-pectin composites have been investigated (Astley et al. 2003, Chanliaud et al. 2002, Chanliaud and Gidley 1999). Chanliaud et al. (2002) demonstrated, through uniaxial tensile testing, that pectin is not the load bearing component of the composite. However, the presence of pectin increased the extensibility of the composite, presumably by interfering with the deposition of the cellulose microfibrils during composite formation (Chanliaud et al. 2002). While not having a load-bearing role in cell walls, pectin affects cell wall porosity and is primarily responsible for the roots cation-exchange capacity (CEC) (Brett and Waldron 1996).

In the short term, binding of Al to the cell wall is important in Al toxicity, with 75 µM Al decreasing soybean root elongation within 5 min (Kopittke et al. 2015) and eventually causing root ruptures (Blamey et al. 2005, Horst et al. 2010, Kopittke et al. 2008). Hence, interaction of Al with the plant cell wall and its constituents is an important early Al response. The binding of Al to the pectin matrix of the cell wall causes rigidification of the cell wall, thereby inhibiting normal elongation processes (Kopittke et al. 2015, Ma et al. 2004, Tabuchi and Matsumoto 2001) and root ruptures may be related to this interference. Studying mechanical and structural properties of plant cell walls is challenging. Individual single cell walls cannot be tested with standard mechanical methods, hence some research into Al toxicity has utilized large, easily cultured algal cell walls (Taylor et al. 2000). Furthermore, whole roots consist of many different types of cells, with the strongest cell walls of the vascular tissue having the most influence on strength measurements. There are limited studies investigating various mechanical properties of whole cell walls or whole roots after exposure to Al (Gunse et al. 1997, Jones et al. 2006, Ma et al. 2004, Tabuchi and Matsumoto 2001). However, these studies do not test, exclusively, Al interaction with pectin, and the resultant effects on the mechanical properties of the cell wall. Interactions with pectin are important as it has been widely accepted that pectin content, and its physio-chemical properties including its' CEC effect on cation binding (Brett and Waldron 1996, Guigues et al. 2014, Willats et al. 2006). In the present study, BC-pectin composites were used as cell wall analogues, providing a readily accessible, homogenous and easily manipulated model system by which to study Al-induced effects on mechanical properties of the

composites. The aim of this experiment was to determine if Al affects the load bearing properties of BC-pectin composites as a model for plant cell walls.

Materials and methods

Pectin preparation

Commercial pectin from citrus fruit was obtained from Sigma Chemical Corporation (St. Louis MO, USA). Pectin was dissolved by slow addition of pectin powder to rapidly stirred TDI water. The resultant 1% solution was stirred for at least 12 h. Subsequently, the pectin solution was stirred for 15 min under CO_2-free air, de-esterified with 1 M KOH and stirred for a further 15 min under CO_2-free air. Thereafter, the H^+ form of Amberlite IR-120 cation exchange resin was added to remove K^+ ions and stirred for 15 min. Centrifugation at 1000 g RCF for 15 min removed the resin. The galacturonic acid (GalA) concentration and degree of esterification (DE) of pure pectin solutions was determined by titrimetric analysis (Walter 1991). Chanliaud and Gidley (1999) achieved optimum production of BC-pectin composites using DE 30% pectin, therefore pectin was prepared to DE 30% and added to the media at the appropriate time.

Bacterial composite preparation and characterisation

Komagataeibacter xylinus strain ATCC 53524 from the American Type Culture Collection (Manassas, VA, USA) was grown in two culture media to form pectin composites (Chanliaud and Gidley 1999). The standard culture medium (HS) was based on that developed by Hestrin and Schram (1954) containing, 5.5 g l^{-1} peptone, 5.5 g l^{-1} yeast extract, 1.27 g l^{-1} citric acid, 3.8 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$ and 2% (w/v) glucose. The HS medium was modified to minimize complexation and precipitation of Al by replacing citric acid and phosphate with potassium hydrogen phthalate (KHP) The modified medium (HS-KHP) was prepared by dissolving 5.5 g l^{-1} peptone, 5.5 g l^{-1} yeast extract, 11.4 g l^{-1} potassium hydrogen phthalate (KHP), 0.16 g l^{-1} NaOH in TDI water. Both media were adjusted to pH 4 with 10% HCl, autoclaved for 15 min at 121°C and cooled to room temperature. Thereafter, DE 30% pectin and 2% glucose were added aseptically. The primary inoculum was prepared by transferring a single colony to 10 ml aliquots of media and 2 ml of 0.125 M $CaCl_2$ in 70 ml sterile specimen jars. Bacteria were grown statically for 3 days at 30°C. The primary inoculums were then shaken on an orbital shaker for 5 min at 400 rpm and combined to form a cell suspension to inoculate experimental replicates. For BC-pectin composite production, 16 ml media, 2 ml primary inoculum, followed by 2 ml 0.125 M $CaCl_2$ under gentle agitation (orbital shaker, 250 rpm for 5 min) were added to a 70 ml sterile specimen jar. Chanliaud and Gidley (1999) had shown that the addition of $CaCl_2$ causes the media to gel, a prerequisite for cellulose to interpenetrate during *K. xylinus* incubation. Each specimen jar with a diameter of 42 mm produces one, circular pellicle floating on the surface of the medium, constituting one replicate. Incubations were performed statically for 96 h, after which composites were harvested and washed under gentle agitation (orbital shaker, 150 rpm for 5 min) in ice-cold 12.5 mM

CaCl$_2$. Composites were stored in sterile specimen jars in 0.2% NaN$_3$ solution at 4°C to inhibit bacterial or fungal degradation.

Pectin incorporation into the pellicle was determined on a dry weight basis using the modified colorimetric assay for galacturonic acid (Blumenkrantz and Asboe-Hansen 1973) after hydrolysis for 1 h in concentrated H$_2$SO$_4$-borate at 0°C. The chromophore 3-phenylphenol was used, and sulfamic acid acted as the colour depressant for neutral sugars (Filisetti-Cozzi and Carpita 1991). The BC-pectin composites consisted of approximately 30% pectin by weight, (data not shown) which is consistent with optimum composite formation (Chanliaud and Gidley 1999).

Aluminium treatment of BC-pectin composites

Beakers containing 500 ml of 12.5 mM CaCl$_2$ and 0, 40, 80 or 160 μM AlCl$_3$ were adjusted to pH 4 with 0.5 M HCl, and cooled to 4°C. Measured concentrations of Ca and Al were used to calculate the activity of Al^{3+} in the treatment solution as 0, 10, 20 or 40 μM using PhreeqcI (Parkhurst 2007) with the phreeqc database. For comparison, Kopittke et al. (2008) had determined that an activity of 16.5 μM Al^{3+} in 1 mM CaCl$_2$ reduced cowpea (*Vigna unguiculata*) root growth by 50%. Individual BC-pectin composites were placed into the beakers and allowed to equilibrate for 16 h at 4°C. For method optimization, composites were removed, blotted on the side of the beaker to remove excess solution, and further blotted for 20 s on a dry filter paper (dry weight recorded). The filter paper was immediately weighed again (wet weight), as was the hydrated pellicle, this procedure being used to determine entrained solution. The composite was then freeze dried and the dry weight recorded. Solutions were sampled again with the final pH determined to have decreased by pH 0.04–0.1. Thereafter dry composites were digested and analysed for Al by inductively coupled plasma mass spectroscopy (ICPMS), Ca by inductively coupled plasma optical emission spectroscopy (ICPOES). Pectin incorporation was determined colorimetrically. Aluminium incorporation into the pellicle was determined on a dry weight basis (Table 1). Once the Al-exchange system was optimized, the exchange treatments were repeated with five replicate composites per treatment. Aluminium-exchange was also performed on a batch of BC-pectin composites grown in the HS-medium at pH 4, (i.e. the same procedure is followed as described above, but citric acid and Na$_2$HPO$_4$·2H$_2$O were used instead of KHP and NaOH, at the quantities stated above) to quantify the effects of the modified medium on cellulose production.

Tensile testing

After equilibration, composites were removed from the beakers and tensile strength assessed by uniaxial testing using an Instron 5543 (Instron, Melbourne, Australia) as described by McKenna et. al. (2009). Briefly, dumbbell shaped strips of pellicle with dimensions of 2.63×14 mm were cut using a dumbbell press (ISO 37-4) and thickness measured on individual samples using digital callipers. Three dumbbells were cut per pellicle and there were five replicates per treatment. The two ends were placed

directly between vice grips, and moved apart at a constant speed of 10 mm min^{-1}. A 5-N load cell was used to record the force required for extension as a function of time. Force-deformation data were converted to stress-strain profiles from the geometrical measurements. Engineering stress (σ) was calculated by F/A where A is the area measured and F is the force in N. Strain (ε) was calculated by $\Delta L/L_o$ where ΔL is exerted extension from the starting point L_o, and converted to a percentage. The apparent Young's modulus, calculated by $(F/A_0)/(\Delta L/L_o)$, was estimated from the average slope over the strain range of 5–15%.

Scanning Electron Microscopy

Samples of BC-pectin composites to be examined by scanning electron microscopy (SEM) were freeze-substituted according to the method of Wharton (1991). Approximately 1 cm^2 sample pieces were frozen in liquid nitrogen for 10 s and immediately transferred to a solution of 3% glutaraldehyde in methanol at –20°C for 24 h. Samples were then transferred to methanol (100%, without glutaraldehyde) at –20°C for a further 24 h, removed from the freezer, allowed to warm to room temperature, and dried using a Balzers critical point drier. Thereafter, the freeze-substituted samples were coated with approximately 10 nm of Pt using an Eiko IB-5 sputter coater and examined using a field emission scanning electron microscope (JEOL JSM 6300F) at 6 kV and 3 to 5 mm working distance.

Results and discussion

Bacterial cellulose-pectin composites grown in the HS-KHP medium contained 40–50% pectin, which was shown by SEM to be closely associated with cellulose microfibrils (Fig. 1). Individual *K. xylinus* cells were visible also. This result is consistent with previous studies optimizing composite formation in the original medium (Chanliaud and Gidley 1999). Furthermore, the 40–50% pectin of the BC-pectin composites was similar to that of primary cell walls with up to one third of cell wall polysaccharides (Willats et al. 2006, Willats et al. 2001) supporting the proposition that the BC-pectin composites are a model for plant cell walls. It is noteworthy that pectin incorporation did not differ among the Al treatments (Table 1).

The three Al treatments resulted in successful incorporation of Al into the composite, with 40 and 160 μM treatments incorporating more Al than the 80 μM treatment (Table 1). Pectin incorporation did not change with Al treatment, owing to the background of $CaCl_2$ preventing dissolution of pectin and the entrained solution was < 10% of the hydrated weight of the pellicle (Table 1). The Al concentration in the entrained solution was unlikely to have any bearing on the mechanical properties of the composites; therefore, rinsing was unnecessary.

Overall treatment effects on individual stress and strain profiles (Fig. 2) indicated the range determined in the BC-pectin composites with average stress and strain values and apparent Young's modulus presented in Table 2. Aluminium treatment of BC-pectin composites, at all concentrations

tested, had no significant effect on the stress or strain values measured in HS or HS-KHP media composites (Table 2). Furthermore, Al had no consistent effect on Young's modulus values of the BC-pectin composites. Higher Young's modulus values were obtained for BC-pectin composites grown in the original HS media, most likely due to optimal microbial metabolism, improving cellulose deposition (Table 2). Significant difference in Young's modulus were seen in the HS media, with the control and 160 µM Al treatments yielding the highest values. The minor differences in stress and Young's modulus values seen between the two growth media is best attributed to natural variation in pellicle formation. Overall, the presence of Al, at the concentrations tested, had no marked effect on the tensile properties of BC-pectin composites (Table 2).

The currently-accepted Type I cell wall model depicts the plant cell wall as containing three structurally independent but interpenetrating networks of cellulose, xyloglucan and pectin (Carpita and Gibeaut 1993, Carpita and McCann 2002, McCann and Roberts 1991). In this model pectin is not considered a 'load-bearing' component; this function is fulfilled by the cellulose-xyloglucan 'scaffolding' network. Cellulose is able to reinforce cell walls under stress by orientating in the direction of stress and it is the mean orientation of cellulose that has been shown to determine wall mechanical properties (Kerstens et al. 2001). Chanliaud et al. (2002), showed, that upon removal of the pectin component of BC-pectate composite, tensile deformation profiles remained largely the same. This result indicates that cellulose is the main contributor to the tensile strength of the composite. However, the presence of pectin modified the arrangement of the cellulose microfibrils as they were being deposited which is analogous to deposition in the plant cell wall (Chanliaud et al. 2002). The results of the present study support the findings that cellulose is the major strength component of these composites as exposure of BC-pectin composites to Al resulted in no changes to either the stress or strain values measured. Therefore, Al-pectin makes no significant contribution to the mechanical strength of the composites and possibly cell walls. In a model system of pectin only, Mimmo et al. (2005), showed, by FT-IR, that the adsorption of Al onto preformed Ca-pectate gels weakened the overall structure of the gel. They concluded that Al showed a weaker interaction with the carboxylic groups and other functional groups of the pectic backbone than Ca, and the regular order of a 100% Ca-pectate bound gel was deformed into disordered chain associations with the addition of Al (Mimmo et al. 2005).

Although Al had no effect on the tensile properties of BC-pectate cell wall analogues, this does not preclude Al having an effect on the mechanical processes involved in cell wall elongation, particularly by modifying cell wall porosity. Our previous study demonstrated that Al decreased hydraulic conductivity of BC-pectate composites by modifying the porosity of the pectin component (McKenna et al. 2010). Changes in pectin porosity are likely to affect cell wall enzyme access (Wehr et al. 2004) and ion transport (Gessa et al. 2005), with probable impacts on cell wall elongation and growth.

There are a small number of studies that investigate the effect of Al on cell wall composition and

the mechanical properties of root cell walls. It has been demonstrated that Al reduced the viscous and elastic extensibility of wheat (*Triticum aestivum*) cell walls (Ma et al. 2004, Tabuchi and Matsumoto 2001). Aluminium-induced inhibition of root elongation has been attributed to rigidification of epidermal cell walls in maize (*Zea mays*) and tomato cell walls (Jones et al. 2006, Kopittke et al. 2015, Postma et al. 2005, Tabuchi and Matsumoto 2001). Levels of cell wall polysaccharides increase in cell walls exposed to Al, potentially inhibiting normal cell wall elongation process (Hossain et al. 2006, Le Van et al. 1994, Nguyen et al. 2005, Tabuchi and Matsumoto 2001). Maize suspension cells adapted to NaCl and DCB, formed cell walls with increased amounts of pectin, leading to greater Al sensitivity, by accumulating more Al and having lower cell viability (Schmohl and Horst 2000). The often contrasting results of studies investigating effects of Al using pectin based systems and those using various plant tissues are likely due to the heterogenous nature of roots comprising a number of cell types which confounds the effect of Al on the cell wall. Furthermore, in this study the DE of pectin (30%) was chosen to optimize composite formation, however in root cells pectin DE varies according to developmental stage ranging from 50–80% (Goldberg et al. 1995, McCann and Roberts 1996) with the degree of esterification known to influence Al binding (Mimmo et al. 2009).

Conclusions

This study investigated the mechanical properties of bacterial cellulose-pectin cell wall analogues treated with Al. Although Al was successfully incorporated in the composite, by binding with the pectic fraction of the composite, Al had no effect on the tensile properties at the concentrations tested. This finding is consistent with pectin not being the main load bearing component of root cell walls. This study describes, for the first time, a detailed methodology by which BC-pectin composites can be applied to research into Al-pectin interactions. This method can overcome the difficulties of investigating plant cell walls with their small cell size and heterogeneous composition. The finding that Al has no effect on the tensile properties of BC-pectin composites suggests that Al has other effects on the mechanical processes involved in cell wall elongation (e.g. by reducing cell wall porosity).

Author contributions

B. McKenna was responsible for experimental design, all experimental work and drafting of the manuscript. D. Mikkelsen was involved in tensile testing experiments, expertise *K. xylinus* fermentation and editing of the manuscript. J. B. Wehr, F. P. C. Blamey and N. W. Menzies were involved in experimental design, academic supervision and editing of the manuscript.

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Figure legends
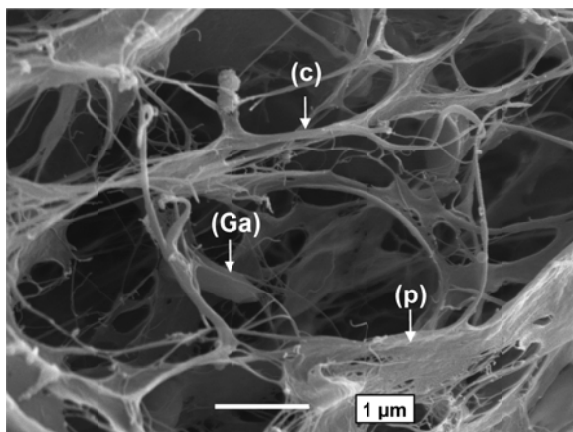
Fig. 1. Micrographs of BC-pectin composite consisting of approximately 30% pectin. The image illustrate *K. xylinus* cells (Ga) and cellulose microfibrils (c) intimately associated with pectin (p).
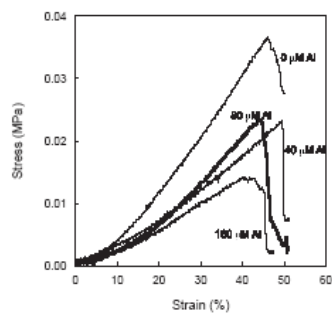
Fig. 2. Example stress versus strain plots for bacterial cellulose (BC)-pectin composites grown in HS-KHP medium and treated with 0 to 160 µ*M* Al.

Table 1. Physical and chemical properties of control bacterial cellulose (BC)-pectin composites and after Al treatments in a matrix of 12.5 mM $CaCl_2$. Values are the mean $\pm$ standard errors (n = 3). *Entrained solution and Al concentrations were not measured on BC-pectin composites that were not treated with Al.

Treatment	Dry weight (mg)	Pectin incorporated (%)	Entrained solution (%)	Al pre exchange (μmol)	Al post exchange (μmol)	Al in whole pellicle (μmol) (digest result)
BC-P	41	49.1 ± 1.60	N/A*	N/A*	N/A*	N/A*
BC-P + 40 μM Al	42	48.5 ±1.76	9.2 ± 2.0	21.2 ± 0.11	20.5 ± 0.31	2.66 ± 3.12
BC-P + 80 μM Al	38	44.5 ± 7.72	9.6 ± 3.2	42.1 ± 0.11	38.6 ± 0.28	1.03 ± 0.24
BC-P + 160 μM Al	39	46.2 ± 6.36	8.1 ± 2.7	82.0 ± 0.39	73.0 ± 0.63	2.08 ± 0.17

Table 2. Mechanical properties of control bacterial cellulose (BC)-pectin composites and after Al treatments in a matrix of 12.5 mM CaCl$_2$. Apparent Young's modulus was calculated over strain of 5–15%. Values are the mean ± standard errors (n = 5). For each media, within a column means (± SE) with the same letter are not significantly different at the 5% level.

Treatment	Tensile Stress (MPa)	Tensile Strain (%)	Apparent Young's Modulus (MPa)
HS Media			
BC-P	0.034 ± 0.003[a]	41.74 ± 3.20[a]	0.071 ± 0.009[a]
BC-P + 40 μM Al	0.056 ± 0.021[a]	49.49 ± 3.08[a]	0.054 ± 0.008[ab]
BC-P + 80 μM Al	0.032 ± 0.003[a]	48.53 ± 3.16[a]	0.042 ± 0.002[b]
BC-P + 160 μM Al	0.039 ± 0.005[a]	44.24 ± 3.32[a]	0.066 ± 0.007[a]
LSD (5%)	NS	NS	0.023
HS-KHP Media			
BC-P	0.034 ± 0.008[a]	41.17 ± 3.10[a]	0.058 ± 0.010[a]
BC-P + 40 μM Al	0.023 ± 0.002[a]	44.83 ± 3.61[a]	0.041 ± 0.006[a]
BC-P + 80 μM Al	0.021 ± 0.003[a]	47.94 ± 3.91[a]	0.029 ± 0.004[a]
BC-P + 160 μM Al	0.026 ± 0.006[a]	46.91 ± 6.99[a]	0.037 ± 0.004[a]
LSD (5%)	NS	NS	NS