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Salt stress on *Lotus tenuis* triggers cell wall polysaccharide changes affecting their digestibility by ruminants

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Authors Contributions

MEV carried out and discussed all the experiments, GJ planned and supervised experiments of nutritional parameters and *in vitro* gas production, MAC planned and supervised LM and TEM analyses, JAZ planned and supervised the plant growth experiments, MC planned and supervised the chemical characterization of polysaccharides, JME and MC discussed and wrote the paper, and JAZ and MC designed the whole investigation. All authors contributed to review the different versions of this paper, as well as the final Manuscript.

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2 digestibility by ruminants**

3

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41 **Highlights**
42
43 Plants of *Lotus tenuis* were affected negatively in their growth by salt stress
44
45 Shoots and leaves produced increased amounts of material soluble in KOH solutions
46
47 KOH extracts from shoots comprised xyloglucans, glucuronoxylans and also pectins
48
49 In some tissues thicker but more opened cell wall structures were found
50
51 Quality as forage improved by increased amounts of more digestible polysaccharides
52
53

54 **Abstract**

55

56 *Lotus tenuis* is a glycophytic forage legume (Fabaceae) used in feeding ruminants that can grow
57 under salinity and waterlogging stresses. Plants obtained in controlled conditions were affected
58 negatively in their growth by the effect of salt. Results from sequential extraction of plant cell
59 wall polysaccharides and chemical characterization were related to those from nutritional
60 parameters used to assess ruminants feedstuffs (Van Soest detergent system). Shoots and leaves
61 were analyzed, and the most important differences were found for shoots. The salt-stressed shoots
62 gave lower values of neutral detergent fiber and acid detergent fiber; they produced higher
63 amounts of reserve α-glucans, and hemicelluloses (xyloglucans and glucuronoxylans from
64 primary and secondary cell walls, respectively) and pectins, leaving less material resistant to
65 extraction. This effect was clearly confirmed by an *in vitro* gas production assay. In addition,
66 observations by light microcopy (LM) and transmission electron microscopy (TEM), showed in
67 some tissues thicker walls and more opened cell wall structures in regard to control samples,
68 which could allow easier access of degrading enzymes in the rumen. Although the plant biomass
69 of *Lotus tenuis* produced under salt stress was lower, its quality as forage improved due to
70 production of increased quantities of more digestible polysaccharides.

71

72 ***Keywords:*** *Lotus tenuis*, cell wall, salt stress, pectin, hemicellulose, forage digestibility

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75 **Abbreviations:**

76 ADF: acid detergent fiber, AIR: alcohol insoluble residue, AGPs: arabinogalactan-proteins, Ara:
77 arabinose, CDTA: 1,2-Cyclohexylenedinitrilotetraacetic acid, Cel: cellulose, cwNDF: netral
78 detergent fiber determined using the residue from the water extraction as starting material, CWP:
79 cell wall polysaccharides, DM: dry matter, e: stomata, EC: electric conductivity, ep: epidermis, f:
80 fibers, fl: floem, FR: final residue, Fuc: fucose, Gal: galactose, GalA: galacturonic acid, GC: gas
81 chromatography, Glc: glucose, GlcA: glucuronic acid, GX: glucuronoxylans, Hem: hemicellulose,
82 HG: homogalacturonan, HSF: two half sibling families, ivDMD*: in vitro* dry matter digestibility,
83 LDA: lignin of acid detergent fiber, LM: light microcopy, m: medulla, M: mannans, NCGP; net
84 cumulative gas production, NDF: neutral detergent fiber, NDFD: neutral detergent fiber
85 digestibility, NMR: nuclear magnetic resonance spectroscopy, pc: cortical parenchyma, RGI:
86 rhamnogalacturonan I, Rha: rhamnose, S: more susceptible family, T: more tolerant family, TEM:
87 transmission electron microscopy, TFA: trifluoroacetic acid, UA: uronic acid, XG: xyloglucans,
88 x: xylem, Xyl: xylose, αADFom: acid detergent fiber with α-amylase and reported ash-free,
89 αNDFom: neutral detergent fiber with α-amylase and reported ash-free, * (Asterisk): cambium,

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91 **1. Introduction**

92 The floodable Salado Basin region of around 9 million hectares on the eastern side of the province
93 of Buenos Aires (Argentina; ~35-38ºS, 57-63ºW). It holds a significant natural and seminatural
94 grasslands area and constitutes one of the most important areas for beef production [1]. *Lotus*
95 *tenuis* is a glycophytic forage legume (Fabaceae) originated in Mediterranean areas and
96 naturalized in many regions of Argentina. It is highly nutritious for grazing cattle, and it
97 predominates in this area due to its ability to grow under salinity and waterlogging stresses [2–5].
98 Perennial pastures, particularly legumes, can reduce groundwater recharge, improve feed value,
99 and help to achieve more sustainable production systems in saline areas [6,7] due to their high
100 digestibility and acceptability, high protein content, and the possibility of nitrogen fixation
101 through the interaction with rhizobium and mycorrhiza.

102 Salt stress is one of the most serious environmental factors limiting plant growth and crop
103 productivity [8]. When plants are suffering salt stress, they usually show growth reduction at two
104 phases: the exogenous phase of water stress, caused by salt accumulation outside roots, and the
105 endogenous phase of injury, caused by inherent salt accumulation [9]. Salt stress can cause
106 multiple adverse effects on plant metabolism, but certain plants have mechanisms of salt tolerance
107 at molecular, cellular, and the whole plant levels [10]. Plant cell walls are dynamic entities that
108 govern the morphology, growth and development of plants [11]. They are composed of a highly
109 integrated and structurally complex network of polysaccharides, including cellulose,
110 hemicelluloses and pectins, as major components [12,13]. Plant cell walls mediate interactions
111 between the environmental stresses and the cell. Cell wall metabolism reacts in response to
112 wounding (*e.g.* mechanical, herbivory) [14,15], cold and heat stress, drought and flooding
113 tolerance, and salt stress [16,17]. The adaptability of cell wall components is, in part, inherently
114 enabled by being constituted by major amounts of carbohydrates. There are more than 10 major
115 sugar residues that are commonly found in plant cell wall components, each of which can occur in
116 multiple forms [18]. Changes in different polysaccharides quantities and structures may have
117 important consequences regarding interactions between them and with other biopolymers, and
118 hence in the cell wall assembly. In recent years, most research on the role of cell walls in abiotic
119 stress has been focused on changes on expression of genes related with cell wall enzymes mainly
120 in mutants of the model plant *Arabidopsis thaliana* [17,19], and also with differences determined
121 *in situ* by multiple advanced microscopic techniques involving visualization of cell wall
122 compounds [20,21], but very few have deepen into changes in polysaccharides structure isolated

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123 from them, which constitutes another possible approach. This is in part due to the experimental
124 difficulties related with structural determination of cell wall polysaccharides, that requires
125 important quantities of biomass, as well as time-consuming procedures.
126 Primary cell walls from legumes belong to Type I [22,23]. They comprise important amounts of
127 pectins, xyloglucans as major hemicellulose, and cellulose, as structural polysaccharides. Pectins
128 constitute a mixture of heterogeneous, highly hydrated polysaccharides rich in α- D-galacturonic
129 acid; they comprise two major constituents, homogalacturonan (HG) and rhamnogalacturonan I
130 (RG I). While HG is a homopolymer of (1→4)- α- D- galacturonic acid, RG I is a rod- like
131 heteropolymer of repeating (1→2)- α- L- Rha- (1→4)- α- D- GalA disaccharide units.
132 Arabinans, galactans, and highly branched arabinogalactans of various structures and sizes, are
133 attached to the *O*- 4 of many of the rhamnose residues of RG I, with a degree of substitution
134 varying with cell type and physiological state [24]. Secondary cell walls are particularly
135 developed in fibers and xylem, and they are constituted by major amounts of cellulose and/or
136 lignin, and glucuronoxylans as major hemicellulose, although xyloglucans are also found in minor
137 quantities. Small amounts of mannans are found in primary and secondary cell walls [25,26]. Due
138 to their functionality, secondary cell walls are more developed in shoot than in leaves tissues. The
139 scarse information available, indicates that in salt-sensitive plants, primary and secondary cell
140 walls are altered by salt stress [15].
141 Availability of nutrients in a feed is essentially determined by its chemical constitution, firstly
142 with respect to the concentrations of available components, and secondly by the organic structures
143 and inhibitors that may constrain the accessibility. From the nutritional viewpoint, digestibility is
144 the proportion of the feed or feed component that is not excreted (it represents the amount of
145 nutrients or energy available for the animal metabolism), and from a physicochemical point of
146 view, it is a function of cumulative availability of net nutrients [27] through the digestion process.
147 Forages represent the single most important feed resource on breeding cattle of the Buenos Aires
148 Salado Basin, hence plant cell walls amounts and digestibilities are an important constrain of
149 ruminants intake, nutrient and energy supply, and herefore ruminant production. Despite the fact
150 that plant cell wall composition and its resulting ultrastructural changes brought about by abiotic
151 stresses could cause a significant impact on the nutritive value, there is a lack of direct
152 information regarding plant cell wall responses to salt stress [28]. Elucidating methods to study
153 the cell wall polysaccharides structures and digestibility of *L. tenuis* grown under controlled salt

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154 stress conditions, similar to those found in Buenos Aires Salado Basin accurately, should allow ~~us~~
155 to predict the potential nutritive value of pastures in the field.
156 In this study, the response of two half sibling families (HSF) of *L. tenuis* with different
157 susceptibility to salt stress [29] were compared to gain insight on the plant physiological status of
158 *L. tenuis* grown under controlled salt stress conditions. Their potential nutritive value as indicated
159 by plant growth, anatomy, and chemical characterization of cell wall and reserve polysaccharides,
160 in relation to their digestibility were analyzed. It was found that although salt stress triggered a
161 reduction in plant biomass of *Lotus tenuis*, its quality as forage improved due to production of
162 increased quantities of more digestible polysaccharides. The results were analyzed taking into
163 account polysaccharides structures and their solubility behavior.
164

165 **2. Materials and Methods**

166 **2.1. Plant Material.** Seeds of *Lotus tenuis (Waldst. and Kit., syn. L. glaber)* from two half sibling
167 families (HSF) with different susceptibility to salt stress were provided by the National Institute of
168 Agricultural Technology (Estación Experimental Agropecuaria Pergamino, INTA, Buenos Aires,
169 Argentina). The HSF 490 family was expected to be more tolerant (T) than the HSF 2241 family
170 classified as more susceptible (S) to salinity. Seeds were scarified by rubbing with fine sand paper
171 (3M P400) and sown in Petri dishes containing two sheets of filter paper. They were watered daily
172 and kept in germination chambers at 25°C (constant temperature), with a 24 h photoperiod
173 (day/night). Light was provided by Grolux fluorescent lamps (F 40 W). Seedlings were transferred
174 to germination trays filled with compost, and kept at controlled temperature (25°C) and light
175 (400W), being daily watered. When roots became visible, plants were transferred to 2.56 l pots of
176 20 cm diameter (5 plants per pot) filled with compost/perlite (3/1 v/v) and placed on trays (5 pots
177 per tray) under greenhouse conditions. Two experiments were conducted on consecutive years
178 with plants of the same HSF, using 1100 plants on the first year, and 110 plants on the second one.
179

180 **2.2. Experimental design and treatment in stress conditions.** The experiment was performed
181 according to a completely randomized design of two factors: HSF (T and S) and salinity dose
182 (control (C), watered without added salts; dose 1, watered with 75 mM NaCl (D1); and dose 2,
183 watered with 150 mM NaCl (D2)). Plants were watered with half saline dose during one week,
184 until the forth pentafoliate leave opened, and after that, with full saline dose, until the appearance
185 of the first flower, when the essays ended. The electric conductivity (EC) on pots was monitored

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186 every 7 days with a conductimeter HI 993310, (Hanna Instruments, Woonsocket, Rhode Island,
187 USA), temperature inside the greenhouse was measured with a 16 channel datalogger
188 Cavadevices, (CABA, Argentina), and solar radiation with a radiometer (Ceptometro LP-80,
189 Decagon Devices, Pullman, Washington, USA). The plant growth variables measured at the end
190 of the experiment included: dry matter of aerial part (shoots and leaves) and roots; biomass of
191 aerial part, and roots; shoot and root length, and number of shoot ramifications. The second
192 experiment carried out in similar conditions gave the same tendencies for these plant growth
193 variables regarding HSF and salt stress conditions (Supplementary Table S1). To simplify
194 presentation, taking into account that treatment with D1 and D2 showed similar tendencies, but in
195 treatment with D2 some differences were more important, only those obtained with D2 are shown,
196 however results obtained with D1 are available as Supplementary material (Supplementary Tables
197 S2-S6).
198
199 **2.3. Anatomical studies by LM and TEM.** For light microscopy (LM), pieces of leaves, shoots,
200 and roots of each treatment of about 1 mm^3 were cut with a scalpel and fixed in 3% (v/v)
201 glutaraldehyde in 0.2 M Na_2HPO_4 - Na_2HPO_4 buffer solution (pH 7.4) at 4 °C for 12 h. Samples
202 were then washed with the buffer for 24 h and postfixed in 1% (w/ v) OsO_4 aqueous solution at
203 20 °C for 2 h. Then, they were washed with the same buffer for 1 h, dehydrated in an ascending
204 ethanol series (50, 70, 90, 100% (v/v), 15 min each) and ethanol-acetone series (2:1, 2:2, 1:3; pure
205 acetone, 15 min each) at 20 °C, and embedded in low viscosity Spurr' s resin [30]. Transverse
206 ultrathin sections (1 µm thick) were stained on slide with 0.05% (w/v) toluidine blue in 2.5%
207 (w/v) Na_2CO_3 solution (pH 11.1) for 1–6 min at 60 °C, and examined under a Leica DM 2500
208 microscope attached to a computer and a digital camera Leica EC3 (Leica, Wetzlar, Germany).
209 All reactives were from Merck Química Argentina S.A. (Argentina). For Transmission electron
210 microscopy (TEM), ultrathin sections (1 µm thick) were cut using a glass knife with a Sorvall MT
211 2-B ultracut microtome, collected on copper grids and double stained with uranyl acetate and
212 Reynolds lead- citrate [31]. Sections were examined using a JEOL JEM 1200 EX II Transmission
213 Electron Microscope (Tokyo, Japan) at an accelerating voltage of 90 kV. Digitised images with
214 webplotdigitizer ©2010-2020 software were used to measured epidermis thickness (µm), shoot
215 cross-section area (μm^2), perimeter shoot cross section (µm), pith cross-section area (μm^2) and
216 pith perimeter area (µm).

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218 **2.4. Nutritional parameters**

219 **2.4.1. General methods.** Shoots and leaves samples were analysed for dry matter (DM) (105°C
220 during 4 h), ash (600°C during 2 h) (AOAC, 1990; #942.05), Neutral detergent fibre (aNDFom)
221 with α-amylase and acid detergent fibre (ADFom) were reported ash-free according to Van Soest
222 *et al.,* 1991 [32], with an ANKOM® equipment (Model 220, ANKOM™ technology, Fairport,
223 NY, USA). Lignin content was obtained by sulfuric acid treatment (LDA). Cellulose (Cel) and
224 hemicellulose (Hem) content were calculated as Hem=aNDFom – ADFom; Cel= ADFom –
225 LDA). In the text, plain abbreviations were used expressed as g/kg DM (NDF and ADF).

226 **2.4.2. *In vitro* gas production technique**. To assess the *in vitro* cumulative gas production**,**
227 fermentation kinetics and digestibility, samples obtained from shoots and leaves cell walls (the
228 residues of the hot water extraction, see below) were incubated in dark brown, 100 mL bottles
229 with a Bromobutyl septa caps (20 mm diameter) and sealed with aluminium caps. Ruminal liquor
230 (~ mass ratio solid: liquid 50:50) was collected before the morning feeding from two cannulated
231 ewes, fed to maintenance with a standard diet alfalfa pellet:maize grain, 70:30. Incubation
232 medium was prepared by mixing one part of ruminal liquor with 10 parts of carbonate-bicarbonate
233 buffer. According to the method of Theodorou *et al*. 1994 [33], modified by Wawrzkiewicz and
234 Danelón, 2004 [34], duplicate samples were incubated during 48 h and 72 h at 39°C. Pressure
235 changes inside the incubation bottles were measured using a pressure transducer (T443A, Bailey
236 and Mackey Ltd, Birmingham, England) connected with a three-ways valve at 2, 4, 6, 9, 12, 17,
237 20, 24, 30, 36, 48 and 72 h of incubation (*i.e.,* twelve times), pressure values were corrected by the
238 amount of substrate DM incubated. Volume was then regressed on pressure records, in order to fit
239 a linear regression model, to calculate the actual volume record for every bottle and time. Raw gas
240 production data were corrected by their respective blanks, in order to calculate net cumulative gas
241 production (NCGP), and data were expressed per grams of incubated DM basis (ml/g DM). By
242 adding 2–3 drops of a saturated thymol solution, the fermentation was terminated after 48 h of
243 incubation on two bottles of each sample/treatment as a first step to evaluate the *in vitro* dry
244 matter digestibility (ivDMD), the remaining bottles continued in the incubating bath until 72 h.
245 Each bottle content was filtered through fiber filter bags (ANKOM #F57) before being sealed.
246 The ivDMD was calculated from the filter bags residues, after being treated with neutral detergent
247 solution, according to Van Soest *et al*. (1966) [35]. Neutral detergent fiber digestibility (NDFD)

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248 was analyzed according to Goering and Van Soest (1970) [36]. True DMD at 48 and 72 h of
249 incubation was therefore calculated as follows: *ivDMD (%)* =100 - *NDF residue* x 100/DM
250 incubated.
251
252 **2.5. Plant cell wall chemical analyses**
253 **2.5.1. Isolation and sequential extraction of cell wall polysaccharides.** Dried and milled
254 samples of shoots and leaves of T plants from *L. tenuis* were extracted separately. The material
255 was sequentially extracted with organic solvents (two times with alcohol, two times with acetone,
256 once with ethyl ether), and then two extractions with hot H_2O for 2 h at 90° C were carried out
257 [37]. After each extraction the residue was separated from the supernatant by centrifugation. The
258 supernatant and the residue were dialyzed and freeze-dried separately. In this way two hot-water
259 extracts were obtained for each material (S-W1 and S-W2, for shoots, and L-W1 and L-W2, for
260 leaves). The residue from the water extraction was sequentially extracted at room temperature
261 with 0.05 M CDTA at pH=6, 0.05 M Na_2CO_3, and 1 M and 4 M KOH solutions (40g /L) in the
262 same way (twice with each solvent, except for 4 M KOH solutions), giving 9 extracts (CDTA1-
263 CDTA2, $Na_2CO_3$1-$Na_2CO_3$2, 1MKOH1-1MKOH2, 4MKOH1-4MKOH2-4MKOH3) and a final
264 residue (FR). After each extraction, the residue was separated from the supernatant by
265 centrifugation (9500 rpm at 4 ºC; Hermle Z 323 K, Germany). Plant cell wall extracts and residues
266 were dialyzed (MWCO 6-8,000) against tap water for 72 h, then against distilled water for further
267 4 h, and finally lyophilized.
268 **2.5.2. Analyses of cell wall extracts.** Total carbohydrate content of extracts was analyzed by the
269 phenol–sulfuric acid method [38]. Total sugar content on residues was determined using a phenol–
270 sulfuric acid method adapted for insoluble material [39]. For the uronic acid (UA) determination,
271 the samples were hydrolyzed using the method of Ahmed and Labavitch (1977) [40] and the
272 uronic acids concentration was determined according to Filisetti-Cozzi and Carpita (1991) [41].
273 The methods of Lehrfeld 1987 [42] and Walters & Hedges 1988 [43], modified by Zelaya et al.,
274 (2017) [44] were used to detect and quantify galacturonic acid, glucuronic acid and 4-*O*-
275 metilglucuronic acid on representative samples, using gas chromatography (GC/MS). To
276 determine the neutral monosaccharide composition using gas chromatography (GC), alditol
277 acetates were obtained by hydrolysis with TFA 2M for 2h at 120ºC, reduction with $NaBH_4$, and
278 acetylation [45]. GC of the derivatized samples, were carried out on an Agilent 7890A gas-liquid
279 chromatograph (Avondale PA, USA) equipped with a flame ionization detector and fitted with a

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280 fused silica column (0.25 mm id × 30 m) WCOT-coated with a 0.20 µm film of SP-2330
281 (Supelco, Bellefonte PA, USA). Chromatography was performed as follows: (a) from 200ºC to
282 240ºC at 2ºC min^{-1} followed by a 15-min hold for alditol acetates; (b) from 235°C to 248ºC at 2ºC
283 min^{-1} followed by a 30-min hold for uronic acids quantitation. N_2 was used as the carrier gas at a
284 flow rate of 1 ml min^{-1} and the split ratio was 80:1. The injector and detector temperature were
285 300ºC. GC–MS of the alditol acetates was performed on an Agilent 7890A gas-liquid
286 chromatograph interfaced to a GCMS QP5977A mass spectrometer. Chromatography was
287 performed on the SP-2330 capillary column as described above, but in this case, He was used as
288 carrier gas at a flow rate of 1.3 ml/min. Mass spectra was recorded over a mass range of 30–500
289 amu. Enzymatic treatment to remove reserve α-glucans was performed on S-W1D2 using α-
290 amylase type VI-B from bovine pancreas (Sigma). A solution of the sample (5 mg/mL) in
291 phosphate buffer 0.1M pH 6.9 was kept 24 h at room temperature with constant agitation, then it
292 was dialyzed and freeze dried. Nuclear Magnetic Resonance spectroscopy (NMR) of selected
293 extracts was carried out. The sample (10 mg), previously exchanged with deuterium by repeated
294 evaporations in D_2O, was dissolved in D_2O (1 mL) and placed in 5-mm tubes. Spectra were
295 recorded at room temperature on a Bruker Avance II 500 spectrometer (Karlsruhe, Germany). For
296 ^{1}H NMR experiments the parameters were: a spectral width of 6.25 kHz, a 76º pulse angle, an
297 acquisition time of 3 s, a relaxation delay of 3 s, for 32 scans. Pulse sequence for ^{1}H-^{13}C HSQC
298 technique was supplied by the spectrometer manufacturer; spectra were recorded at room
299 temperature and they were obtained at a base frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C.
300 Signals were referenced to internal acetone at 2.21 ppm for ^{1}H NMR and 31.1 ppm for ^{13}C NMR
301 experiments, respectively.
302

303 **3. Results and Discussion**

304 **3.1. Effect of salt stress on plant growth.** *Lotus tenuis* grown under salt stress conditions (150
305 mM NaCl, dose 2) had 70% overall biomass reduction (as dry weight, $p<0.001$), shorter shoots
306 with lower number of ramifications (54% and 68.1% in regard to controls, $p<0.0001$) and lower
307 percentages of dry matter and biomass of roots, as well as root length (40% ($p=0.02$), 30%
308 ($p=0.001$), and 40% ($p=0.001$), respectively) (Figure 1A, B, Supplementary Figure S1). Although
309 salt stress affected similarly T and S half sibling families (HSF) of *L. tenuis* in a negative way
310 with respect to these variables, T plants presented higher values for each parameter analyzed.
311 Growth reduction by salt stress was previously found for field and laboratory experiments with *L.*

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312 *tenuis* and *L. corniculatus* [4,7,8,44–46], as well as for soybean [47,48]. No visible differences
313 were found between HSF by light microscopy (LM), but salt stress induced several structural
314 differences between control and treated plants (Figure 1C). Shoots from salt-treated plants
315 exhibited smaller cross section overall area versus control (216.4 mm^2 vs 297.1 mm^2), thinner
316 cortex (74.9 µm vs 101.5 µm), visible diameter reduction in xylem vessel elements, and cell wall
317 width increase in vessel elements and fibers. In addition, thickness increase of the outer tangential
318 epidermal cell wall was also detected on leaflets and roots of stressed specimens (data not shown).
319 In agreement, important changes in plants growing under 150 mM NaCl stress condition were
320 detected at the transmission electron microscopy (TEM) level on transverse shoot sections (Figure
321 1D). Cell walls appeared thin, compact and electron dense suggesting a possible alteration in cell
322 wall polysaccharides (hemicellulose-cellulose) distribution pattern. Likewise, the accentuated
323 electron density of middle lamellae could be related with a pectic polysaccharides increment [46].
324 Fibers appeared grouped in bundles and showed conspicuously thicker walls in regard to control
325 shoots, constituted by apposition of successive concentric lamellae electronically translucent.
326 Reduced lumens and electronically dense middle lamellae were also observed. Similar anatomical
327 changes due to salt stress, including an increment on thickness on epidermal cell walls of shoots
328 were reported previously [47,48]. The reduction in the diameter of the vessel elements in shoots
329 could be interpreted as an adaptive response of *L. tenuis* to the conditions imposed by the increase
330 of salt in the irrigation water [48], as has been proven in other crops, such as *Phaseolus vulgaris*
331 and *Panicum antidotale* [49,50]. These strategies contribute to prevent embolism risk ensuring the
332 continuity and efficiency of water conduction [51–53], which increase plant survivorship to salt
333 stress conditions.
334
335 **3.2. Salt stress impact on nutritional parameters.** Since salt-treatment had profound impact on
336 plant growth and cell wall thickness, it is worthwhile asking if these morphological changes
337 translate into changes in the fiber content of the biomass. Indeed, the NDF and ADF of shoots
338 (Figure 2A) decreased with increasing NaCl concentration ($p<0.01$; for both HSF, a NDF 536 for
339 control and 415 g/kg DM for shoots of salt stressed T plants). In addition, the salt stress caused a
340 20% ADF reduction, while the amount of hemicellulose and cellulose decreased with salt stress,
341 no significant differences were detected for lignin (LDA, Figure 2A). Hence, the increase in
342 salinity induced significant changes in detergent fiber parameters of shoots with potential
343 nutritional consequences, while in leaves, it only decreased significantly ADF (see below). Saline

344 stress was previously associated with reductions in NDF and ADF [54], for aerial parts (shoots
345 and leaves) of *L. corniculatus*, as well as for other legumes. Ben-Ghedalia et al (2001)[55]
346 reported for *L. multiflorum, Lam.* that salt concentration in water was positively associated with a
347 decrease in NDF and an increase in soluble carbohydrates content, which should comprise not
348 only reserve oligo- and polysaccharides, and low molecular weight carbohydrates, but also cell
349 wall carbohydrates, like pectins and highly soluble hemicelluloses. Previous studies showed that
350 carbohydrates, such as glucose, fructose, sucrose, and starch accumulated under salt stress
351 conditions [28,47,56–58]. In this study, no differences were observed for LDA of shoots in
352 association with salt stress, contrarily to previous reports in which increased vascular cells
353 thickness was usually attributed to an increased lignification dependent on salt concentration [59].
354 For example, salt stress was positively associated with lignin concentration in soybean shoots due
355 to deposition in the vascular system or further development of the xylem, which turned out in a
356 lower growth [60]. Forage consumption and digestibility are highly dependent on cell wall
357 abundance, proportion of tissues of different degradability, chemical composition, lignin
358 proportion and other physicochemical properties that impact on cell wall digestibility at ruminal
359 level.
360
361 **3.3. Impact of salt-stress on the *in vitro* gas production of shoots cell walls of *L. tenuis*.** In
362 order to determine the impact of plant changes induced by salt on *in vitro* digestibility of cell
363 walls, the residue from the hot water extraction were used for the experiments. The net cumulative
364 gas production (NCGP) in the first 15 h of incubation of the plant cell walls was 18% higher for
365 plants watered with 150 mM NaCl than for control, with the highest difference in hourly gas
366 production rate (*ca.* 52%) at the fifth hour (Figure 3). Between 20 and 40 h of incubation, the
367 hourly gas production rate showed little differences between cell walls of treated plants and
368 control. The *in vitro* dry matter digestibility (ivDMD) of shoots after 48 h of incubation increased
369 in those samples collected from plants watered with high levels of salt (for control, 567 g/kg and
370 for 150 mM NaCl, 646 g/kg DM) (Supplementary Figure S3). The low ADF, and high ivDMD
371 and *in vitro* gas production determined in this study could be expected for increased cell wall
372 polysaccharide degradability, and hence improved forage quality. These results are in accordance
373 with structural and chemical changes found for shoots of plants watered with saline solutions with
374 respect to control plants (see below).
375

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376 **3.4. Characterization of cell wall polysaccharides from shoots of *L. tenuis* under salt stress.**
377 Considering the changes found in fiber content, as well as in determinations of *in vitro*
378 digestibility of the salt-treated biomass, the chemical nature of these modifications at the cell wall
379 were explored. Since preliminar chemical analyses carried out on cell walls from shoots of both, S
380 and T families of HSF showed similar results (results not shown) and T plants had better yields in
381 biomass than those described as S, T plants were used for full cell wall carbohydrate
382 characterization. The alcohol insoluble residue (AIR) from shoots of control (C) and treated plants
383 with 150 mM solutions of NaCl (dose 2, D2) were sequentially extracted with hot water, with
384 aqueous solutions of 0.05M CDTA, 0.05M Na_2CO_3, and 1M and 4M KOH at room temperature
385 (Figure 2B). The overall yield of carbohydrate material from neutral or moderately alkaline
386 extracts (S-W1,2, S-CDTA1,2, and S-$Na_2CO_3$1,2) was lower for shoots of salt-stressed plants than
387 for those of control plants, while yields for highly alkaline extracts (S-1MKOH1,2 and
388 4MKOH1,2) were much higher (Figure 2C). This yield increase of 74% of extracts obtained with
389 KOH solutions was so important, that the plant material in the final residue (FR) was smaller for
390 the salt stressed plants.
391 Reserve polysaccharides represented a high proportion of the hot water soluble polysaccharides
392 (S-W1; Table 1), while cell wall polysaccharides comprised mainly homogalacturonans (HG) and
393 rhamnogalacturonan I (RG I) (Tables 1 and 2). Important numerical differences were found
394 between treatments. In extracts from shoots of salt-treated plants, the amount of reserve
395 polysaccharides increased greatly, while the total amount of cell wall polysaccharides decreased
396 when compared to control. In addition, differences were found in the polysaccharide structures
397 between the stressed and control plants, evidenced by an increase in arabinose in the former and a
398 concomitant decrease in galacturonic acid (Table 1). In summary, in salt stressed plants, the
399 amount of the water soluble pectins decreased, and the structure of RG I was modified due to
400 higher degree of ramification with arabinose or longer arabinose chains.
401 In order to look deeper into the structure of water-soluble cell wall polysaccharides, extract S-
402 W1D2 was studied by NMR spectroscopy (Figure 4A, B). The sample contained significant
403 amounts of 4-linked α-glucans [61], but some structural details about cell wall polysaccharides
404 were also evident. As expected, important signals corresponding arabinose units were present. In
405 particular, 5-linked, 3-linked, 3,5-linked, and terminal + (2-, 2,3- and/or 2,3,5)-linked α-L-
406 arabinofuranose units were present in molar ratio 45:22:18:15 (as calculated by integration of the

407 signals at δ_H 5.01, 5.08, 5.10, and 5.17 in the ^{1}H NMR spectrum). Also, 4-linked β-D-
408 galactopyranose units, and partially esterified α-D-galacturonic acid units were clear. Extract
409 SW1-D2 was submitted to treatment with α-amylase and NMR analysis of the modified sample
410 (Figure 4C), in which the amount of glucose fell from 61% to 15% of the total neutral
411 monosaccharides, confirmed the assignment of pectin structures and showed additionally the
412 presence of 3,6-linked β-D-galactans, as well as small amounts of xylans. A signal at $\delta_{H/C}$
413 53.9/3.73 was assigned to galacturonic acid residue having the carboxylic acid residues as methyl
414 esters [62]. These results confirm the correct assignment of the monosaccharide composition to
415 the different polysaccharides performed in this study.
416
417 In order to solubilize cell wall pectins, the residues from the water extraction were further
418 extracted sequentially with aqueous solutions of CDTA and Na_2CO_3 (Figure 2B). Major quantities
419 of HG were detected in all these extracts, which should correspond to calcium-linked and
420 covalently linked pectins, respectively, in view of the extraction solvents used to obtain them [37].
421 Extracts obtained with CDTA solutions still had small amounts of α-glucans, but in those obtained
422 with aqueous sodium carbonate, and in all the following extracts, glucose was considered to
423 derive from cell wall polysaccharides. Taken together, the yields of these extracts were very
424 significant, comprising 85 and 74 g/kg DM for control plants and those having 150 mM NaCl in
425 the irrigation water, respectively. Noteworthy is that comparing yields obtained with both solvents
426 these polysaccharides were distributed differently, for control shoots 65% was obtained with
427 CDTA, indicating Ca^{2+} binding to the cell wall, while for salt stressed plants 67% was obtained
428 with sodium carbonate solutions. These results, together with the differences in yields of the hot
429 water extracts, showed that salt stress modified not only the yields of the extracts, but also the
430 extraction patterns, which suggest different assembly in the cell walls. The residues from the
431 extraction with 0.05M Na_2CO_3 aqueous solutions were sequentially extracted with highly alkaline
432 solutions, two extractions with 1M KOH and two extractions with 4M KOH. The residues of the
433 extraction procedure were analyzed, and it was found that cellulose was the major polysaccharide
434 (55-60 moles% of glucose), but they still contained significant amounts of uronic acids (20-30
435 moles%), which should derive from pectins (Table 1). Hence, a third extraction with 4M KOH
436 and analysis of the residues did not show important differences regarding the previous ones, so it
437 was assumed that the extraction was exhaustive for the conditions used here (Table 1). These

438 results showed the presence of important amounts of cellulose as expected, but also some pectins
439 extremely resistant to highly alkaline solutions.
440 Analysis of the monosaccharide composition of extracts obtained from shoots with 1M KOH
441 showed that they were constituted by major quantities of hemicelluloses, in agreement with their
442 extraction conditions, although small amounts of pectins were also present (Table 1). Moreover,
443 for S-1M KOH1D2, analysis of the uronic acids composition showed 22.5% of 4-*O*-
444 methylglucuronic acid, 4.7% of glucuronic acid, and only 3.6% of galacturonic acid. This cell
445 wall derived extract also contained important quantities of xylose (45.8 %) and glucose (16.1 %).
446 The results indicated the presence of both xyloglucans and glucuronoxylans. The existence of
447 glucuronoxylans was proved by NMR spectroscopy (Figure 4D), which showed signals
448 corresponding to unsubstituted and 2-substituted 4-linked β-D-xylopyranose and terminal α-D-
449 glucuronic acid residues (anomeric signals at $\delta_{C/H}$ 102.6/4.41, 102.4/4.55, and 98.5/5.21,
450 respectively, [63]). The first extracts obtained with 1M KOH from shoots were the richest in
451 hemicelluloses, and there was an important difference in the composition of control (S-
452 1MKOH1C) and stressed plants (S-1MKOH1D2) samples. S-1MKOH1C showed smaller
453 amounts of xyloglucans, which should derive from primary cell walls, in regard to S-1MKOH1D2
454 which had major quantities of glucuronoxylans, deriving from secondary cell walls (Table 2).
455 Similarly, xyloglucans were also found in lesser amounts in control from the second extract
456 obtained with the 1M KOH (S-1MKOH2C), than in that from stressed plants (Supplementary
457 Table S4). Monosaccharide composition of extracts obtained with 4M KOH showed an increase in
458 arabinose and, in the first two extracts, also in uronic acids and rhamnose, and a decrease in
459 xylose due to salt stress, indicating an increase in pectin polymers and in xyloglucans and a
460 decrease in glucuronoxylans (Tables 1 and 2, and Supplementary Table S4). The results indicated
461 that xyloglucans were produced in more important quantities by stressed plants, and that there
462 were pectins strongly linked to the cell wall, and their amount increased with the salt stress. As
463 previously shown, the total amount of material extracted with KOH solutions was higher for the
464 salt stressed samples (Tables 1 and 2). This difference was principally due to the higher yield of
465 the first extract obtained with 1M KOH, consequently, to the increase in hemicelluloses derived
466 from secondary cell walls.
467 In order to further relate the results obtained in this sequential extraction of cell wall
468 polysaccharides (Figure 2B) with nutritional parameters, a determination of NDF was carried out

469 using the residue from the water extraction as starting material (cwNDF), in which the small
470 molecular weight carbohydrates, as well as reserve and small amounts of highly soluble cell wall
471 polysaccharides were not present (Tables 1 and 2, Supplementary Tables S3 and S4). An
472 important decrease was obtained for shoots of salt stressed plants regarding controls (703 g/kg and
473 773 g/kgDM, respectively), showing that not only reserve polysaccharides and pectins were
474 soluble in NDF, but also the cell wall material, extracted in this work with strong alkaline
475 solutions. Moreover, comparison of the results from this determination with the yields of the
476 extraction sequence (Figure 2B) indicated that salt stress increased greatly the solubility of
477 hemicelluloses in neutral detergent, giving increased digestibility of these polysaccharides. The
478 sequential extraction procedure carried out here allowed us to give a detailed picture of the
479 different polysaccharides types and their solubility behavior, contributing to understand the
480 nutritional determinations as a function of the polysaccharides structures and their possible
481 interactions.
482
483 **3.5. Effect of salt stress in leaves.** Although the same experiments were performed with shoots
484 and leaves, the differences found between control and salt-stressed samples from leaves were less
485 important. In leaves only ADF decreased significantly (NDF and ADF were 169 and 161 g/kg DM
486 for control leaves, and 171 and 112 g/kg DM for D2, respectively) (Supplementary Figure S2).
487 Our results indicated that hemicelluloses content was very low in leaves from control plants, as
488 defined by Van Soest [27,64]. In agreement, *in vitro* gas production was similar for plants watered
489 with 150 mM NaCl than for control in the first 7.5 h with a maximum production at about 5 h
490 (Supplementary Figure S4). Between 7.5 and 22 h of incubation, gas production was higher for
491 cell walls from leaves of salt-stressed plants, than of those of control. However, dry matter
492 digestibility (ivDMD) after 48 h did not show significant changes due to salt stress (for control,
493 885, and for 150 mM NaCl, 918 g/kgDM, Supplementary Figure S3).
494 Comparing the amount of reserve α-glucans extracted from leaves and shoots, less quantity was
495 produced by leaves, but there was an increase of 70% for stressed plants, in regard to control (12
496 and 20 g/kg DM of T, for leaves of control and D2, respectively, Supplementary Table S5). Also,
497 lesser amounts of cell wall polysaccharides were obtained by extractions with neutral/moderately
498 alkaline solutions in leaves (92 and 86 g/kg DM, for control and D2, respectively Supplementary
499 Tables S5 and S6) than in shoots. Conversely, high amounts of cell wall material were obtained
500 with highly alkaline solutions (561 and 566 g/kg DM, for control and D2, respectively,

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501 Supplementary Table S6), giving an overall yield of extracted material much higher for leaves
502 than for shoots, distributed in important quantities in extracts obtained with both KOH
503 concentrations (1 and 4M) (Supplementary Figure S5). This result showed that cell walls from
504 leaves had very important quantities of hemicelluloses, which were soluble in neutral detergent,
505 indicating that the definition of hemicellulose by the method of Van Soest should be considered
506 with great care, as previously found for other forages [65]. Almost no differences due to salt stress
507 in yields of the polysaccharide obtained in the extraction sequence were found (Supplementary
508 Figure S5). Although polysaccharide structures of leaves were similar to those found in shoots,
509 there were quantitative differences between organs. Leaves produced higher amounts of RGI in
510 the water extracts and major amounts of xyloglucans in the extracts obtained with KOH solutions,
511 but no evidences of glucuronoxylans were detected, indicating predominance of primary cell wall
512 material (Supplementary Tables S5 and S6). Important amounts of pectins were found in all the
513 extracts and also in the final residues of leaves, as in shoots. In summary, as expected the
514 influence of salt stress on cell wall polysaccharides of leaves was not as evident as in shoots of *L.*
515 *tenuis*. However, cwNDF determined using the residues from the water extraction as starting
516 material showed an important decrease for leaves of salt stressed plants in comparison to controls
517 (339 g/kg and 410 g/kg, respectively). In agreement, *in vitro* gas production also showed that cell
518 wall polysaccharides from salt stressed leaves were more digestible than controls, but after longer
519 incubation times than in shoots, suggesting weaker interactions between hemicelluloses
520 (xyloglucans) and pectins solubilized with KOH solutions during the extraction sequence.
521

522 **4. Conclusions**

523 This study demonstrated that plants of *Lotus tenuis* grown under salt stress were smaller, but
524 comprised more digestible carbohydrates, mainly in shoots. Analysis of NDF of the residue from
525 the water extraction, as well as those from *in vitro* gas production and ivDMD, proved that they
526 contain more digestible cell wall polysaccharides. The secuencial extraction to study the cell wall
527 polysaccharides types obtained with the different solvents allowed us to get more accurate and
528 reliable results to explain the digestibility of the plant material grown in different conditions.
529 Chemical analyses of the different fractions of polysaccharides from shoots obtained by extraction
530 with aqueous solvents showed an increase of the amount of reserve α-glucans with salt stress. A
531 similar effect was found in leaves. Conversely, the amount of pectins obtained in the neutral and
532 moderately alkaline extracts (hot water, CDTA and Na_2CO_3 solutions), which comprised mainly

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533 homogalacturonans, decreased in shoots from plants grown under salt stress. On the other hand,
534 the amount of polysaccharides obtained from shoots with highly alkaline solutions increased with
535 salt stress. In particular, in the first extract obtained with 1M KOH, the amounts of xyloglucans
536 (XG) and glucuronoxylans (GX) increased markedly. In addition, in the first two extracts obtained
537 with 4M KOH, there was a decrease in the amount of GX, while highly resistant pectins were
538 obtained in increased quantities. These results could be related to the increase in electrodensity of
539 the epidermal cell wall and in cell wall width of fibers detected by TEM. It was also shown that
540 although cell walls were thicker in some shoot tissues from salt stressed plants, this should be at
541 least in part, consequence of more opened cell wall structures, which should be more accessible to
542 degradation by the ruminal enzymes. This fact could be related to the higher total yields of
543 polysaccharides extracted, and also to the increase in the amounts of more soluble polysaccharide
544 types. It is important to consider that nutritional parameters indicated similar amounts of lignin for
545 control and salt-stressed shoots. Regarding the cell wall polysaccharides, pectins, mainly
546 homogalacturonans with different solubility behavior were found in all the extracts, in those
547 obtained with water, solutions of CDTA and Na_2CO_3, they were the major components, but also in
548 the alkaline extracts and even in the final residue of the extraction sequence. They represent an
549 important part of the total polysaccharides; the same was found in leaves. This multidisciplinary
550 approach allowed us to prove with no doubt the differences between cell walls in shoots from
551 plants grown under salt stress for this legume in the experimental conditions employed. Further
552 studies should be carried out to generalize these results.
553

554 **Declaration of competing interests**: The authors have no competing interests to declare.
555

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566 **Authors Contributions**

567 MEV carried out and discussed all the experiments, GJ planned and supervised experiments of
568 nutritional parameters and *in vitro* gas production, MAC planned and supervised LM and TEM
569 analyses, JAZ planned and supervised the plant growth experiments, MC planned and supervised
570 the chemical characterization of polysaccharides, JME and MC discussed and wrote the paper,
571 and JAZ and MC designed the whole investigation. All authors contributed to review the different
572 versions of this paper, as well as the final Manuscript.

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Table 1. Yields and analyses of major cell wall extracts and final residue (FR) obtained from shoots of *L. tenuis* control plants (C) and watered with 150mM NaCl (D2)[a].

Extract[b]	Treatment[c]	α-glucans g/kg	CWP[d] g/kg DM	Monosaccharide composition (Moles %)							
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA
S-W1	C	14	33	4.9	**-**	**12.2**[e]	2.2	3.4	14.4	**-**	**62.9**
	D2	22	28	4.5	**-**	**25.4**	2.9	2.4	14.3	**-**	**50.6**
S-CDTA1	C	0.7	**38**	0.6	-	7.2	0.2	-	1.6	-	**90.3**
	D2	1.8	**19**	1.8	-	10.5	0.5	-	6.1	-	**80.3**
S-$Na_2CO_3$1	C	-	**22**	2.1	-	0.4	6.6	1.4	4.3	1.6	**84.2**
	D2	-	**31**	-	-	-	-	-	-	-	**100**
S-1MKOH1	C	-	**66**	1.0	-	3.0	**32.8**	0.2	1.8	**4.0**	**57.2**
	D2	-	**133**	1.0	-	3.9	**45.8**	-	2.1	**16.1**	**31.0**
S-4MKOH1	C	-	**37**	2.5	1.5	**12.6**	**35.5**	0.8	7.8	9.5	29.6
	D2	-	**71**	3.9	1.4	**19.3**	**22.6**	0.2	7.0	11.1	34.5
S-FR	C	-	**326**	0.4	0.1	1.8	4.3	4.4	2.3	**55.2**	**31.4**
	D2	-	**240**	0.4	0.1	2.2	4.0	4.4	2.9	**62.4**	**23.7**

[a]Each cell wall extract was analyzed by determination of total neutral carbohydrates and uronic acids contents, and the composition in neutral monosaccharides. These results were analyzed taking into account the following assumptions: 1) Glucose derived from reserve α-glucans in extracts obtained with hot water and with solutions of CDTA. This was proved by NMR analysis of these extracts, which showed clearly peaks corresponding to 4-linked α-glucopyranose units, and no evidence of β-glucopyranose units (see text). In extracts obtained with alkaline solvents, glucose corresponds mainly to xyloglucans, while in S-FR, to cellulose. 2) All other monosaccharides detected derived from water-soluble cell wall polysaccharides. 3) The only uronic acid present was galacturonic acid in the first extracts, while in highly alkaline extracts, variable quantities of galacturonic and glucuronic acids were present, as determined by analysis of the uronic acid composition (Zelaya et al. 2017). Only analysis of the first extract obtained with each solvent and of the final residue are shown. Complete analysis of other extracts is given as Supplementary Tables S3 and S4. For nomenclature, see Figure 2. [b]S-W1, first hot water extract; S-CDTA1 first extract obtained with 0.05M CDTA aqueous solution at pH 6; first extract with 0.05 M Na_2CO_3 aqueous solution; S-KOH1M 1 first extract obtained with 1M KOH aqueous solution; S-KOH4M 1 first extract obtained with 4M KOH aqueous solution; S-FR, final residue of the extraction procedure. [c]C, control plants; D2, plants watered with 150mM NaCl. [d]CWP, cell wall polysaccharides. [e]Values with important differences between treatments are highlighted.

Table 2. Estimated contribution of different polysaccharides to the main cell wall extracts and the final residue (FR) obtained from shoots from control (C) and watered plants of *L. tenuis* with 150mM NaCl (D2).

Cell Wall Extract	Treatment	**Polysaccharide type**[a] (g/kg of shoots)				
		XG/Cellulose[2]	**GX**	**M**	**HG**	**RGI**
S-W1	C	**-**	0-1	1	**19**	12[3]
	D2	**-**	0-1	-	**13**	14[3]
S-CDTA1	C	**-**	**-**	-	**34**	4
	D2	**-**	**-**	-	**15**	4
S-$Na_2CO_3$1	C	**-**	2	-	**18**	2
	D2	**-**	**-**	-	**31**	**-**
S-1MKOH1	C	**3-5**	**19-24**	-	34	**5**
	D2	**21-43**	**40-65**	-	37-39	**11**
S-4MKOH1	C	**4-7**	10-14	**-**	**8-10**	**10**
	D2	**8-16**	6-17	-	**20-21**	**24**
S-FR	C	**179-196**	0-16	13	**98**	16
	D2	**149-158**	0-19	10	**55**	14

[a]XG=xyloglucans, the ranges correspond to nonsubstituted-completely substituted structures. GX=glucuronoxylans, ranges correspond to the cases in which xylose corresponds to XG, and to the assumption of 10% maximum substitution with GlcA. M=mannans. HG=homogalacturonan, calculated from the total amount of UA minus the amount of Rha, the range corresponds to the possible contribution of GlcA to the total UA content. RGI=rhamnogalacturonan I, calculated as the amount of 2xRha+Ara+ Gal. [2]XG for all the extracts, cellulose only in the final residue, that could also comprise small amounts of XG. [3]Arabinose and galactose in this extract could arise from AGPs.

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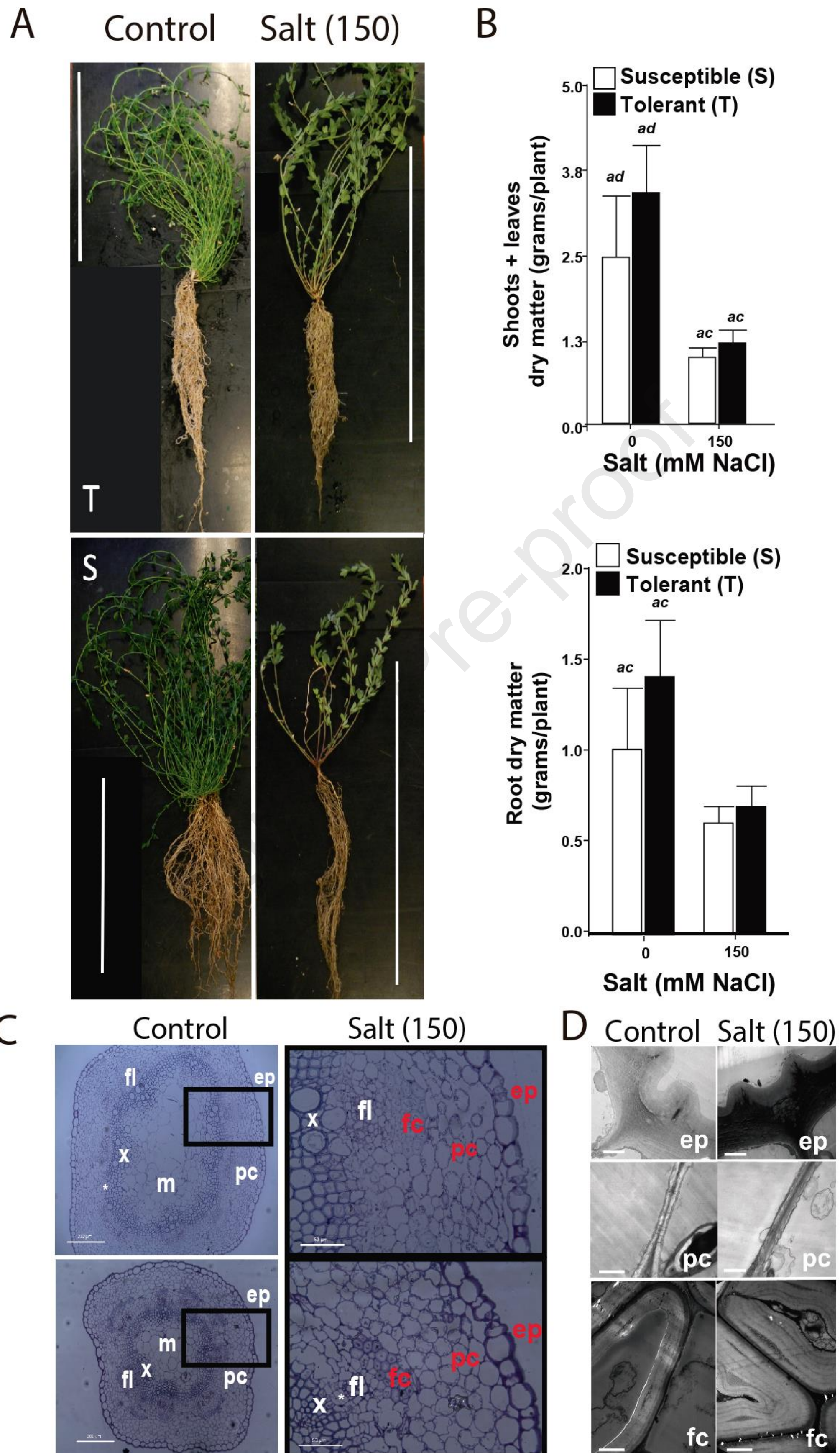

A
Control
Salt (150)
T
S
B
Susceptible (S)
Tolerant (T)
Shoots + leaves dry matter (grams/plant)
5.0
3.8
2.5
1.3
0.0
ad
ad
ac
ac
0
150
Salt (mM NaCl)
Susceptible (S)
Tolerant (T)
Root dry matter (grams/plant)
2.0
1.5
1.0
0.5
0.0
ac
ac
0
150
Salt (mM NaCl)
C
Control
Salt (150)
fl
ep
x
m
pc
fc
D
Control
Salt (150)
ep
pc
fc

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Figure 1. Growth, organ and cellular responses of *Lotus tenuis* plants under salt tress. (**A**) General aspect of plants of *Lotus tenuis* with different susceptibility to salt stress for each saline treatment. Control and treated (with 150 mM NaCl) plants on a more tolerant (T) and a more susceptible (S) variety. (**B**) Dry matter from shoots (on top) and roots (bottom) in control (0 mM NaCl) and salt treated (150 mM NaCl) plants. Bars on the columns represent means ± S.E. Bars followed by different letters differ significantly ($p<0.05$). The first letter is associated with the genotype and the second letter with the salt treatment. (**C**) Micrograph partial views of transverse sections of shoots. Control shoot and treated shoot (with 150 mM NaCl). On the left, general aspect, on the right zoomed areas. ep: epidermis cells; fc: fiber cells; fl: floem; m: medulla; pc: cortical parenchyma cells; x: xylem. Scale 200 µm (on the left) and 50 µm (on the right). Plant cells analyzed by TEM in D are indicated in red. (**D**) TEM micrographs of selected cell types from shoots. Epidermal (ep), parenchyma (pc) and fiber cells (fc) of control shoots (on the left), and the same cell types of shoot-treated with 150 mM NaCl (Salt 150), respectively. Scale =300 nm.

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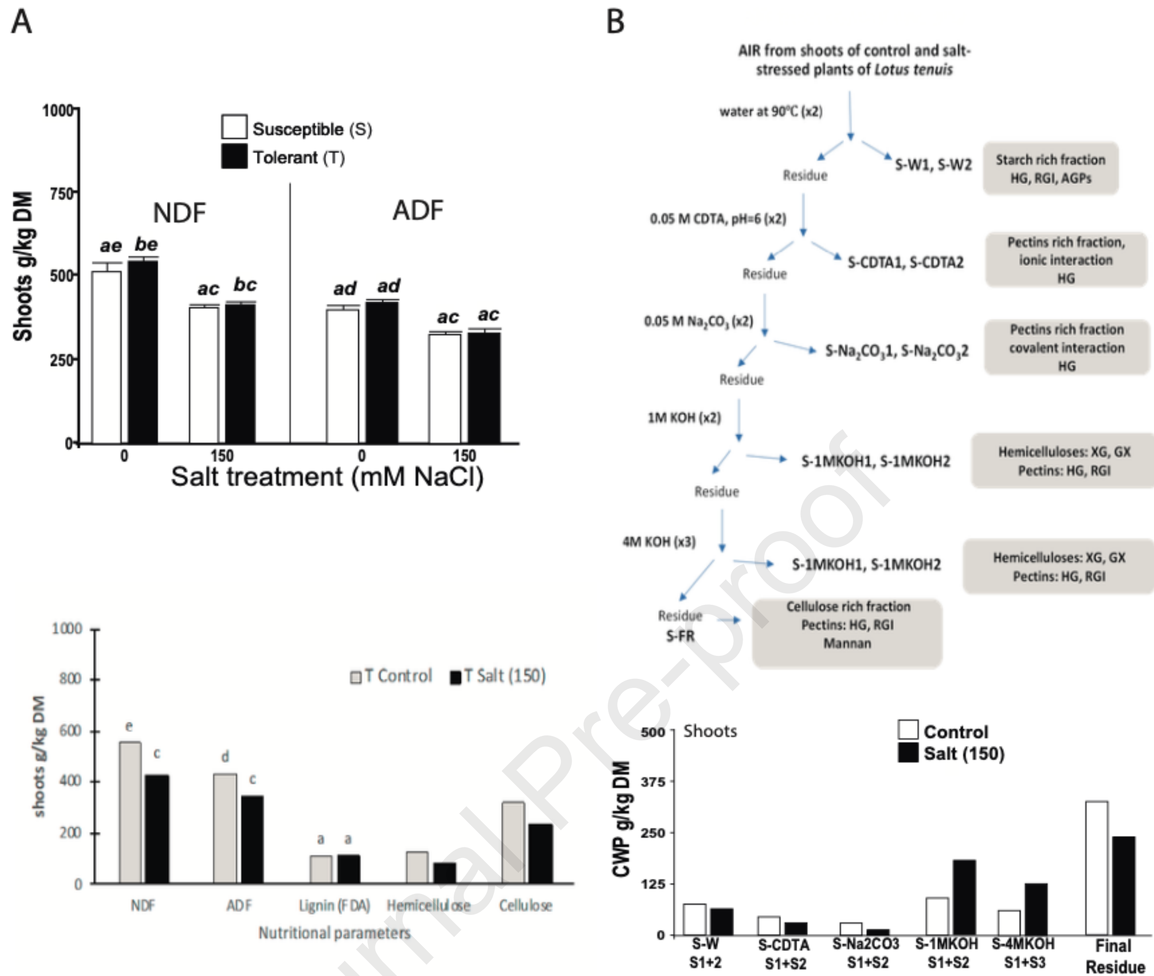

Figure 2. Nutritional parameters and cell wall chemical extracts obtained from shoots of *Lotus tenuis* under salt stress. (**A**) Neutral detergent fiber (g of NDF/kg of DM) and Acid detergent fiber (g of ADF/kg of DM).Variables measured on two half seembling families (T and S). Bars on the columns represent means ±S.E. Bars followed by different letters differ significantly ($p<0.05$). The first letter is associated with the genotype, and the second letter with the salt dose used. On the bottom, nutritional parameters (NDF, ADF, and lignin) for T plants, and the amounts of hemicellulose and cellulose deduced from them (expressed as g/kg of DM). (**B**) Cell wall extracts and final residue obtained by the extraction sequence from AIR. Extractions were carried out at room temperature, unless otherwise indicated. Gray boxes indicate the major polysaccharides detected in each extract. HG: homogalacturonan, RGI: rhamnogalacturonan I, AGPs: arabinogalactan proteins, XG: xyloglucan, GX: glucuronoxylan. On the bottom, overall yields obtained with each cell wall extraction solvent.

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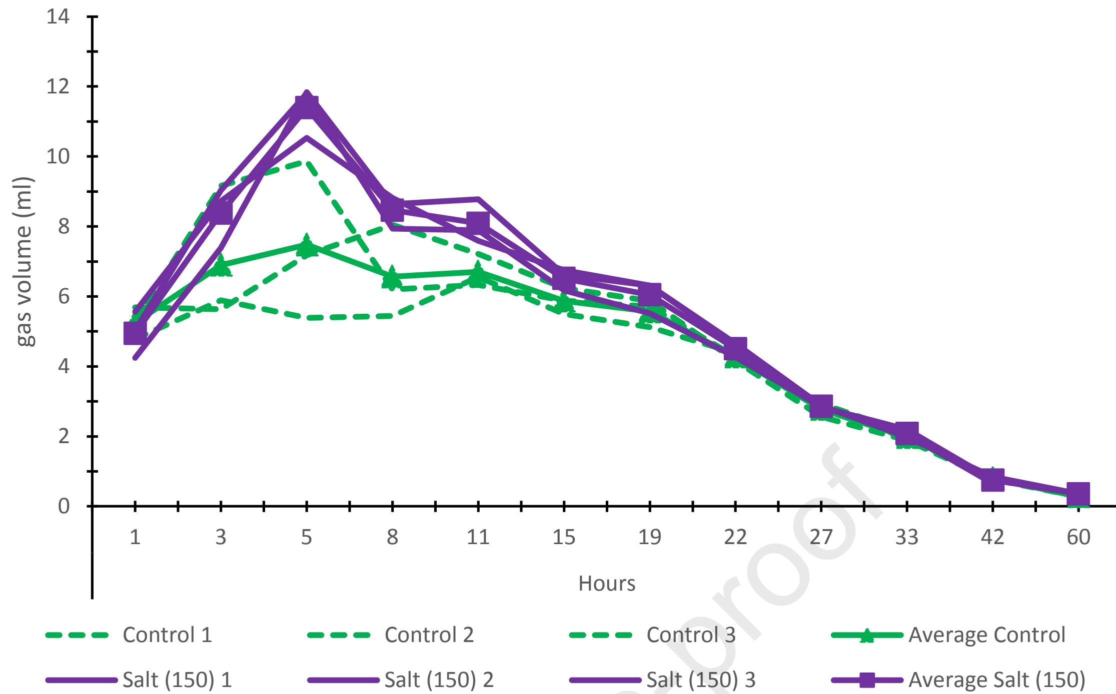

Figure 3. Salt-stress treatment increases the *in vitro* cumulative gas production kinetics from cell wall dry matter of shoots in *Lotus tenuis*. In this experiment, the residue from the water extraction was used as starting material.

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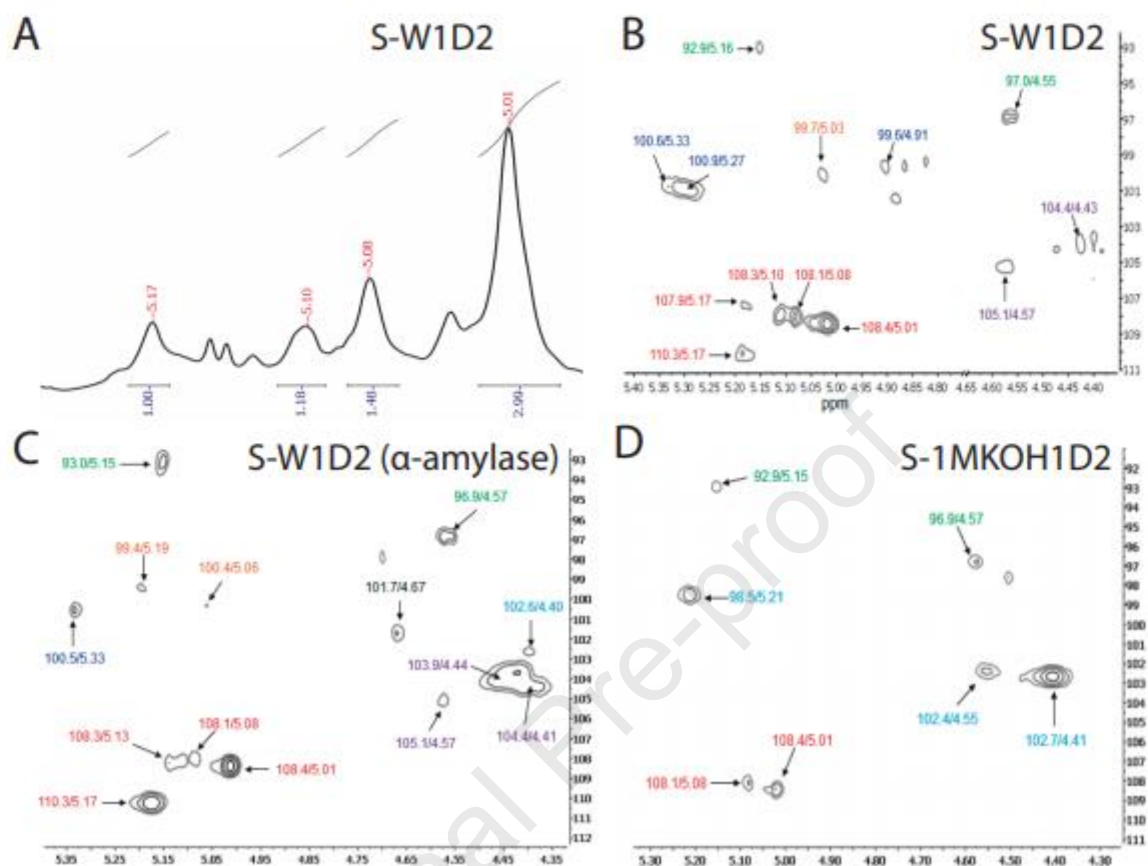

Figure 4. Selected NMR spectra of cell wall extracts isolated from shoots. (A) Anomeric region of the ^{1}H NMR spectrum of S-W1D2 showing integration of the signals corresponding to the different α-L-arabinofuranose residues. (B)-(D) Anomeric region of the HSQC NMR spectra of (B) S-W1D2, (C) S-W1D2 after treatment with α-amylase, and (D) S-1MKOH1D2, showing the signals corresponding to Ara (red), reserve glucans (blue), terminal reducing ends (green), galactans (violet), xylans (light blue), pectin backbones (orange). Signal assignment carried out based on the literature [61-63]

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Highlights

Plants of *Lotus tenuis* were affected negatively in their growth by salt stress

Shoots and leaves produced increased amounts of material soluble in KOH solutions

KOH extracts from shoots comprised xyloglucans, glucuronoxylans and also pectins

In some tissues thicker but more opened cell wall structures were found

Quality as forage improved by increased amounts of more digestible polysaccharides

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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