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Wheat plants sense substrate volume and root density to pro-actively modulate shoot growth

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Running head: Exudate signalling and substrate volume responses

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Abstract: Plants must carefully coordinate their growth and development with respect to prevailing environmental conditions. To do this, plants can use a range of nutritional and non-nutritional information that allows them to pro-actively modulate their growth to avoid resource limitations. As is well-known to gardeners and horticulturists alike, substrate volume strongly influences plant growth, and may be a key source of non-nutritional information for plants. However, the mechanisms by which these substrate volume effects occur remain unclear. Here, we show that wheat plants pro-actively modulate their shoot growth with respect to substrate volume, independent of nutrient availability. We show that these effects occur in two phases; in the first phase, the dilution of a mobile 'substrate volume-sensing signal' (SVS) allow plants to match their shoot (but not root) growth to the total size of the substrate, irrespective of how much of this they can occupy with their roots. In the second phase, the dilution of a less mobile 'root density-sensing signal' (RDS) allows plants to match root growth to actual rooting volume, with corresponding effects on shoot growth. We show that the effects of soil volume and plant density are largely interchangeable, and that plants may use both SVS and RDS to detect their neighbours and to integrate growth responses to both volume and the presence of neighbours. Our work demonstrates the remarkable ability of plants to make pro-active decisions about their growth, and has implications for mitigating the effects of dense sowing of crops in agricultural practise.

Keywords: Soil volume, pot size, plant-plant interactions, neighbour detection, root exudates

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INTRODUCTION

The life of a plant is strongly intertwined with its immediate environment, and particularly the availability of resources (light, water, mineral nutrients). As such, plants must integrate information from their environment, and use this to modulate their growth and development. In vascular plants, the root and shoot systems fulfil completely opposite and mutually interdependent functions in resource acquisition. Roots are dependent on shoots for fixed carbon from photosynthesis, and shoots are dependent on roots for the supply of mineral nutrients and water. Thus, vascular plants must not only integrate information from the environment, but distribute this information within the plant body, and coordinate resulting growth responses over long distances (Wheeldon & Bennett, 2020). Understanding how plants perform these complex balancing acts in order to grow optimally is a key challenge in plant developmental biology.

One obvious solution is for plants to use resource availability to directly or indirectly guide growth. Early physiological models assumed that plant growth was directly influenced by resource availability or limitation (Molisch, 1929) and there has been some recent interest in these types of models (Martín-Fontecha et al., 2018; Barbier et al., 2019). However, while it is certainly true that plant growth can be limited by lack of resources, it is generally a poor strategy for a plant to maximise its growth until resources become limited. Thus, plants also use resource availability to indirectly guide their growth, allowing them to pro-actively modulate their growth to avoid resource limitations (Walker & Bennett, 2018). This is most obvious in the case of mineral nutrients, particular nitrogen (typically in the form of nitrate, N) and phosphorous (typically in the form of phosphate, P), which are the most important nutrients that plants must obtain from the soil. Plants detect the availability of N and P, and use this to regulate the growth of both the root and shoot system through hormonal signalling. The presence of N, P, sulphur and iron in the soil promotes the synthesis of *trans*-Zeatin type cytokinins (*tZ*) (Takei et al., 2001; Takei et al., 2004; Hirose et al., 2008; Seguela et al., 2008; Poitout et al., 2018), while deficiency in P promotes the synthesis of strigolactones (SLs) (Yoneyama et al. 2007; Umehara et al., 2010). Both *tZ* and SLs are transported from root-to-shoot (Hirose et al., 2008; Kohlen et al., 2011; Xie et al., 2015) where they strongly influence a range of parameters such as

29 shoot meristem activity, leaf size and shape, leaf senescence shoot branch number and stem
30 elongation (Wheeldon & Bennett, 2020). This allows plants to match the overall rate of shoot growth
31 to the availability of mineral nutrients, so that growth can be sustained in the long term.
32
33 Plants can also use a wide range of non-nutritional information to guide their growth. This non-
34 nutritional information is particularly useful where it allows plants to 'predict' future resource scarcity,
35 even when current resources are abundant. Again, this allows plants to pro-actively limit growth to
36 avoid resource limitations (Walker & Bennett, 2018). The light reflected by neighbouring plants
37 provides an excellent example of such non-nutritional information. Light reflected from leaves has a
38 much lower ratio of red:far red wavelengths than unreflected sunlight, due to absorbance of red light
39 for photosynthesis (Ballaré & Pierik, 2017). Thus, plants can use the enrichment of far red light to
40 detect the presence of neighbouring plants, and to predict future competition for light (Roig-Villanova
41 & Martínez-García, 2016). In response, they can modulate their growth, restricting branching and
42 promoting stem elongation to outgrow the competing plant. This response does not require active
43 shading, and plants can detect neighbours over some distance via light cues (Roig-Villanova &
44 Martínez-García, 2016). Indeed a 'spike' of far red light is sufficient to induce neighbour detection
45 responses without any difference in the intensity of photosynthetically active wavelengths (Xie et al.,
46 2020). Thus, anticipation of future light limitation is sufficient to modulate growth, in the absence of
47 any underlying resource limitations.
48
49 Besides the availability of mineral nutrients, there are other physical and biological components of
50 the rhizosphere that plants could use to predict the likelihood of resource availability in the future.
51 Limiting the substrate volume available to a plant (usually achieved with a plant pot) has a very
52 strong effect on shoot growth (McConaughey & Bazazz, 1991; Hess & de Kroon, 2006; Poorter at
53 al, 2012). This 'volume restriction' phenomenon has previously been investigated across a range of
54 species including bean, cotton and tomato (Carmi & Heuer, 1981; Gurevitch et al., 1990; Bar-Tal et
55 al., 1995; Xu et al., 2001; Yong et al., 2010). These volume restriction effects are also not caused
56 by water deficit (Krizek et al., 1985; Ismail & Davies, 1998) and indeed, still occur in hydroponic
57 conditions (Ternesi et al., 1994; Bar-Tal et al., 1995; Shi et al., 2008); nor are they correlated with

58 reduced photosynthetic capacity (Carmi & Heuer, 1981; Kharkina et al., 1999; Shi et al., 2008; Yong
59 et al., 2010). Furthermore, although it difficult to experimentally separate the effects of substrate
60 volume and nutrient availability, it is generally accepted that volume restriction is not caused by the
61 availability of nutrients in the substrate (Hess & de Kroon, 2006), a view supported by a meta-
62 analysis of 65 different studies (Poorter et al., 2012). Most notably, using a hydroponic system, Bar-
63 Tal et al (1995) showed that the growth of tomato plants was proportional to the size of the permeable
64 bag they were grown inside, even though they had access to same total volume of nutrient solution.
65 Various factors have been proposed to explain volume restriction effects, including mechanical
66 interactions with pot walls, or the increased heat that occurs in small pots (Poorter et al., 2012), while
67 Falik et al (2005) and Semchenko et al (2007) suggested that self-inhibitory root exudates might
68 account for the changes in root growth that occur in the presence of obstacles and or in limited soil
69 volumes. Currently however, the mechanism by which substrate volume effects occur remains
70 unclear.
71
72 We hypothesise that plants use substrate volume as a form of information to dynamically regulate
73 their growth. That is to say, we hypothesise that plants do not simply grow maximally until they fill
74 their pot, but that they respond to substrate volume early in development to match their growth to
75 the size of the pot. In this study, we tested this idea in hexaploid bread wheat, and attempted to
76 understand how these soil volume effects arise. We show that soil volume effects on shoot growth
77 are indeed proactive, and that they are unlikely to arise from interactions with pot walls. Rather, our
78 data support a model in which plants release and detect two different signals into the rhizosphere;
79 by detecting the increasing concentration of these signals during different phases of growth, they
80 are able to 'measure' substrate volume. Moreover, we show that detection of these signal may also
81 strongly contribute to the detection of neighbouring plants, and allow plants to integrate both soil
82 volume and neighbour density effects into a coherent response.
83

84 # MATERIALS & METHODS

85

86 ## Plant growth conditions and materials

87 For figures 1, 2, 3, 4 and 6, plants were grown on Petersfield No. 2 compost, in greenhouses with
88 supplemental LED lighting to an average intensity of ~250μmol/m^2s^{-1}., on a 16hr day/8 hr night cycle,
89 with a temperature of 20°C. For figure 5, a hydroponic system was used, as detailed below, with the
90 same lighting and temperature conditions. For all experiments spring wheat variety Mulika was used.

91

92 ## Phenotypic assessments

93 Tillering was assessed weekly for all experiments, as a reliable, non-destructive indicator of shoot
94 growth. Other phenotypic measurements, including dry biomass measurements, were generally
95 made at the end of life. For some experiments, we separated wheat biomass into straw and ear
96 biomasses, and then measured seed biomass by threshing the seed from the ears.

97

98 ## Root density quantification

99 Photographs of visible root growth on the four sides and base of clear-sided pots were used to
100 calculate approximate root densities. Images were taken on the same day of each week, every week
101 for 8 weeks. Images were processed using Image J to adjust the contrast and brightness of each
102 image, to increase the signal-to-noise ratio. Every image from the sides of the pots from the same
103 week was treated in the same fashion, but images from different weeks were treated slightly different,
104 reflecting the different lighting conditions on different days. The bases of the pots, which have much
105 higher root densities, were processed separately from the sides, with different adjustments to
106 contrast/brightness - but each image from the bases of the pots from the same week was treated in
107 the same fashion. A pixel containing root will appear nearly white and have a high pixel intensity
108 (scaled from 0-255), while a pixel containing soil will appear nearly black and have a very low pixel
109 intensity. Thus, the mean pixel intensity provides a good indication of the visible root density in each
110 image. We reasoned that the visible root density across the four sides and base of the pot in turn
111 provides a reliable proxy for of the density of roots in the pot as whole. Thus, the pixel intensities

112 were measured for each image were measured using Image J, and then converted to percentages
113 (i.e. mean pixel intensity/255 x 100). The percentages from the four sides and bases added together
114 to give a 'root density score' (RD score) for each pot, which was then averaged across each
115 treatment, for each week.
116

117 Experimental design

118 *Figure 1 and Figure 2*

119 The experiment described in figure 1 was performed with two varieties of spring wheat (Mulika and
120 Willow), two varieties of spring barley and two varieties of spring oilseed rape. Only the Mulika data
121 a described here, but the results were highly comparable for all species.
122

123 Plants were grown in compost in pots containing either 100ml, 500ml or 2000ml of soil, under well-
124 illuminated and well-watered conditions (see above). Half of the plants in each group received 10ml
125 of additional nutrient solution, once per week. We used Arabidopsis Thaliana Salts (ATS) (Wilson et
126 al, 1990) as a standard modular fertiliser. We calculated this to be the equivalent of plants receiving
127 approximately 150kg per hectare of nitrate fertilizer over their lifetime (i.e. in line with fertilizer rates
128 applied to agricultural crops). We tracked tiller number every week for 16 weeks. At the end of life
129 (16 weeks), we measured shoot size using a number of parameters including shoot dry biomass,
130 peak number of tillers, ear number, ear biomass, spikelet number, seed number and seed biomass.
131

132 *Figure 3*

133 We grew wheat plants in compost in clear-walled containers of two different sizes (100ml and 300ml)
134 for 8 weeks. Each week, we photographed each face of every pot, to capture the visible roots.
135 Images were processed as described above. We also tracked the production of tillers in plants
136 across these 8 weeks.
137

138

139 *Figure 4*

We grew five groups of wheat plants in 100ml pots for 4 weeks. For two of these groups (treatments B and E), we made ‘inner pots’ of flexible 35µM nylon mesh shaped to the same dimensions as the 100ml pot. The pots for these two groups had 1cm^2 holes cut into each side-wall of the pot before inserting the mesh inner pot. These pots were then filled with compost. For the other three groups (treatments A, C and D), the unaltered pots were filled with compost as normal. After 4 weeks, plants were then shifted to new growth regimes. Treatment A and B plants continued to be grown in their 100ml pots (unaltered and with a nylon mesh, respectively). Treatment C plants were removed from the 100ml pot, and transferred (with an intact soil/root ball) into a 2000ml pot containing 1900ml of fresh compost. Treatment D plants had 1cm^2 holes cut into each side-wall of the pot, and were then transferred, complete with pot, into a 2000ml pot containing 1900ml of fresh compost. Treatment E plants were transferred, inside their mesh inner pot (but with the outer 100ml pot discarded), into a 2000ml pot containing 1900ml of fresh compost. All treatments were grown for a further 7 weeks. The number of tillers was measured in each plant across the experiment. At the end of the experiment, all plants were removed from their pots and the root system assessed. For 3/10 treatment E plants, their roots had escaped the mesh inner pot, and these plants were not included in the analysis. In the other 7/10 plants, there was no rooting outside the mesh pot, and the outer soil volume was completely unbound.

Figure 5

We grew wheat plants using a hydroponic system. Plants were germinated and grown for 1 week in a 50:50 sand/perlite mix. Equal sized plants were selected, roots were washed and plants were transferred into 1L vessels with lids. To ensure plants were stable within the pot, 50ml centrifuge tubes were used. For the control treatment, 50ml centrifuge tubes were shortened to ~2cm below the screw top lid. For the restrained treatment the bottom ~2cm of the falcon tube was removed and a 35µm diameter nylon mesh was glued across the opening to seal the opening. The centrifuge tube lids had a hole made in them to allow the plant to be passed through it. A foam bung was placed around the shoot root junction and then the lid was tightened on to the centrifuge tube. The lid of the 1L pots had a hole the size of the centrifuge tube made in them to allow the wheat plants in the centrifuge treatments tube to sit stably within the vessel in it. Each pot was filled with 1 litre ATS

169 solution, and water levels were topped up every 2 days. Additional nutrient solution was provided
170 every 2 weeks. Two aquatic pumps (All Pond Solutions, AP-12-Kit Pump) provided aeration via
171 tubing connected to air stones in each pot.

172

173 Root growth was checked daily, and if roots escaped from the restrained treatment tubes, the plants
174 were thrown out. Tillering was recorded weekly for all plants. After 6 weeks, three plants from each
175 treatment were sacrificed to record dry root and shoot biomass. After 8 weeks the experiment ended,
176 and final tiller numbers were recorded, along with the dry root and shoot biomass of all remaining
177 plants.

178

179 *Figure 6*

180 We grew wheat plants at two densities (1/pot and 4/pot) in two soil volumes (100ml and 500ml). To
181 reduce the confounding effects of shoot-mediated crowding, plants were sown within the same
182 surface area in both 4/pot treatments (Figure 6A), and were staked together throughout their lives –
183 effectively forcing the same level of shoot crowding irrespective of soil volume. Tillering was recorded
184 each week for every plant. At the end of the experiment dry shoot biomass was recorded for each
185 plant.

186

187 **Statistical analysis**

188 The sample size used for each experiment is stated in the figure legends. Where multiple plants
189 were grown in the same pot, each sample is one pot; data were averaged within the pot prior to
190 statistical analysis. Data was tested for normality to determine the statistical test most suitable
191 for each experiment.

192

193 RESULTS

194 Shoot growth responds to soil volume independently of nutrient level

195 To define how soil volume influences wheat shoot growth in our conditions, we grew a spring variety
196 (Mulika) in pots containing either 100ml, 500ml or 2000ml of soil, under well-illuminated and well-
197 watered conditions. We observed that the size of the shoot system was clearly proportional to the
198 size of the pot (Figure 1A). We measured shoot size using a number of parameters including shoot
199 dry biomass, peak number of tillers, ear number, ear biomass, spikelet number, seed number and
200 seed biomass. For every parameter, we clearly observed a linear, direct proportionality between pot
201 size and shoot system size (Figure 1B-G; Supplementary Table 1). Thus, as previously
202 demonstrated, soil volume clearly acts as a direct constraint on shoot system size (Poorter et al.,
203 2012). In addition, we treated half the plants in each pot size with additional nutrient solution.
204 However, we did not observe any obvious increase in shoot system size parameter in plants treated
205 with additional nutrients relative to control plants (Figure 1). Thus, consistent with previous studies,
206 our data clearly show that the effect of soil volume on wheat growth is not a nutritional effect (Bar-
207 Tal & Pressman, 1996; Poorter et al., 2012).

208

209 Shoot responses to soil volume are pro-active

210 It was notable that all plants in these experiments proceeded successfully to physiological maturity
211 without any obvious indicators of stress or nutrient deficiency. We therefore hypothesised that the
212 shoot response to volume restriction occurs proactively, early in the life cycle, rather than being a
213 reactive response to stresses caused by limited volume. The growth habit of wheat provided an
214 excellent method for testing this, because wheat plants continually initiate basal branches (tillers)
215 from very early in the life-cycle until flowering, after which non-flowering tillers senesce off. Tracking
216 tiller initiation and senescence rate thus provides a clear temporal insight into changes in overall
217 shoot growth and plant status, without destructive sampling. We tracked tiller number in Mulika plants
218 grown in 100ml, 500ml and 2000ml pots over 16 weeks. In all plants, tillering began after 3 weeks,
219 and we observed almost immediate divergence in tiller production between pot sizes (Figure 2A).
220 This divergence thus represents the point at which the plants detect the limitations of their substrate

221 volume, and modulate their shoot architecture in response. However, this is not the point at which
222 plants stop tillering, which occurs 2-4 weeks later, even in the smallest soil volume. Furthermore,
223 even when tillering stops, plants clearly continue to grow and develop until the normal end of their
224 life (Figure 1A). Differences in tillering at 3 weeks cannot reasonably be explained by nutrient or
225 water limitation, especially when all plants continue growing beyond this point. Moreover, plants
226 provided with weekly additional fertiliser did not have different growth curves relative to untreated
227 plants (Figure 2A).

228

229 Early volume responses do not correlate with root density or mechanical 230 impedance

231 To explain this very early detection of volume restriction, we hypothesised that plants might either
232 sense the mechanical interaction of roots with the pot walls, or sense the increasing density of roots
233 in the container. To examine these ideas, and assess the early effects of volume restriction on root
234 growth, we grew wheat plants (Mulika) in soil in clear-walled containers of two different sizes (100ml
235 and 300ml), and tracked root growth (as visualised through the pot walls) and shoot growth. We
236 observed that, within the first week after germination, roots had already collided with the walls of
237 both pot sizes (Figure 3A), and deflected their growth along the walls. Tillering between the groups
238 diverged between 3 and 4 weeks after germination, and ceased in both groups after 4 weeks (Figure
239 3B). At the start of this critical window, root density was much higher in 100ml pots than in 300ml
240 pots, and continued to rise in both groups throughout the week 4 and into week 5 (Figure 3C). Thus,
241 the cessation of tillering in both groups after 4 weeks did not correlate with mechanical impedance
242 or root density (both higher in 100ml pots), nor did it correlate with any change in root growth (which
243 continued on the same trajectory in both treatments until at least week 5) . However, later changes
244 in shoot growth, namely the senescence of tillers after week 6, was much stronger in the 100ml
245 group (Figure 3B), which correlated with the cessation of visible root growth in this group, while root
246 growth in the 300ml group still continued, albeit more slowly (Figure 3C). These data suggest that
247 early responses to volume restriction do not involve root density or mechanical signalling, and only
248 cause changes in shoot growth, and not root growth.

249 ## Volume responses do not require volume occupation

250 To further understand the genesis of substrate volume responses, we performed an experiment in
251 which wheat roots were constrained within a larger soil volume. In this experiment we can distinguish
252 between substrate volume and rooting volume (i.e. the area actually occupied by roots). We grew 5
253 groups of wheat plants (Mulika) in 100ml pots; 2 of these groups had an additional nylon mesh 'inner
254 pot'. All plants were grown in the 100ml pots for 4 weeks, up to the point they started to become
255 inhibited by the limited soil volume. One group of plants subsequently remained in the 100ml pots
256 (treatment A). One group were removed from their 100ml pots and transferred (with the soil/root ball
257 intact) to 2000ml pots filled with soil (treatment C). One group were kept in their 100ml pots, but
258 with a $1cm^2$ hole cut into each wall of the pot; the plant was then transferred with its pot into a 2000ml
259 pot filled with soil (treatment D). One group of plants grown within the nylon mesh were then left to
260 grow (treatment B), while the other group were transferred with their mesh inner pot (but with the
261 100ml outer pot discarded) into a 2000ml pot filled with soil (treatment E). Treatment A and B plants
262 thus have a substrate and rooting volume of 100ml of soil; treatment C and D plants have a substrate
263 and rooting volume of 2000ml of soil, although with much more mechanical impedance in the case
264 of D, while treatment E plants have a rooting volume of 100ml, but substrate volume of 2000ml
265 (Figure 4A).

266

267 As expected from previous experiments, treatments A and B produced very few additional tillers after
268 week 4, remaining inhibited by the limited soil volume (Figure 4B). Conversely, tillering was very
269 strongly promoted in treatment C plants after transfer, to a level that would be expected from plants
270 grown in 2000ml pots (Figure 4B). Tillering was also strongly promoted in treatment D plants, with
271 the additional mechanical impedance having almost no effect on the growth of plants compared to
272 treatment C (Figure 4B). Intriguingly, despite having no physical access to the additional soil volume,
273 treatment E plants tillered much more vigorously than either treatment A and B plants, although not
274 as strongly as treatment C or D plants (Figure 4B, Figure 4C). The differences in tillering established
275 between the treatments in weeks 4-7 carried over into equivalent differences in the final shoot
276 biomass of the plants (Figure 4D).

277

278 We then performed a modified version of this experiment using a hydroponic system. We grew wheat
279 plants in nutrient solution in aerated 1 litre containers, with the nutrients replenished every 2 weeks.
280 We used two treatments; the root systems were either allowed to freely explore the substrate volume,
281 or were restrained inside a 50ml centrifuge tube, in which the bottom of the tube had been removed,
282 and replaced with a nylon mesh to allow (relatively) free diffusion of nutrients and root exudates
283 (Figure 5A). The 'restrained' plants have the same substrate volume as the control plants, but a
284 much smaller rooting volume, with higher and more immediate mechanical stress. In a preliminary
285 experiment, we had established that plants grown hydroponically in an unmodified 50ml centrifuge
286 tube produced only a single shoot, and their growth was severely impaired.

287

288 We observed that unrestrained plants tillered freely throughout the experiment, producing even more
289 tillers than soil-grown plants in 2000ml pots (Figure 5B). Their shoot and root biomass also increased
290 strongly through the experiment (Figure 5C, D). The restrained plants continued to tiller throughout
291 the experiment, but at a slower rate than unrestrained plants; with a clear difference in rate emerging
292 after 4 weeks when the unrestrained plants strongly accelerated their tillering (Figure 5B). The same
293 trend was also observed for root and shoot biomass throughout the experiment (Figure 5C, D).
294 Between week 6 and week 8, root and shoot biomass in the restrained plants approximately doubled,
295 whereas in unrestrained plants shoot biomass increased ~6 fold, and root biomass ~4. From the
296 midpoint in the experiment, the root systems of the restrained plants were much smaller than the
297 unrestrained plants (Figure 5B), which likely contributed to the later differences between the two
298 treatments.

299

300 Taken together, the results of these two experiments are not consistent with plants being solely
301 limited by rooting volume and/or mechanical stimulus. In both experiments, physically restrained
302 plants with chemical access to the full substrate volume were much bigger than those without
303 chemical access. The results are thus consistent with plants using a chemical signal to detect the
304 substrate volume. This can pass through nylon mesh and be diluted, even if roots are not physically
305 able to escape restraint.

306

307 Shoot responses to soil volume and crowding are partly interchangeable

308 As an alternative way of testing these ideas, we grew multiple plants together in the same substrate
309 volume. This means that each plant has the same absolute substrate volume, and mechanical
310 impedance, but higher root density and higher concentrations of any chemical signal released by the
311 root systems. We grew wheat plants at two densities (1/pot and 4/pot) in two substrate volumes
312 (100ml and 500ml)(Figure 6A). To reduce the confounding effects of shoot-mediated neighbour
313 detection, plants were sown within the same surface area in both 4/pot treatments (Figure 6A), and
314 were staked together throughout their lives.

315

316 In the 1/pot treatments, as expected based on previous experiments, the plants grew according to
317 their soil volume and were 4.0-fold larger in 500ml pots than in 100ml pots in terms of shoot biomass
318 (Figure 6C). Conversely, plants grown at a rate of 4/pot in 500ml were 2.9-fold smaller than plants
319 grown 1/pot (Figure 6C). Thus, a 5-fold increase in pot volume increased shoot growth by 4-fold (a
320 response ratio of 0.8), and a 4-fold increase in plant density decreased shoot growth by 2.9x-fold (a
321 response ratio of 0.72). This suggests that the effects of plant density and soil volume are
322 qualitatively similar, and the mean substrate volume (soil volume/number of plants) available to each
323 plant strongly predicted the shoot growth of the plants. Consistent with this, plants grown 4/pot in
324 500ml were 3.2-fold larger than plants 4/pot in 100ml pots (Figure 6C). While the effect is slightly
325 less than the 4-fold effect of increasing soil volume in 1/pot plants, increasing soil volume clearly
326 largely alleviates the effect of increasing plant density, suggesting that neighbour detection and
327 volume sensing may be at least partially integrated through the same mechanism.

328

329 DISCUSSION

330 A mobile, non-nutritional 'substrate volume-sensing signal' drives early 331 volume responses

332 Although it is well established that nutrient levels do not explain substrate volume responses (Figure
333 1; Hess & de Kroon, 2006; Poorter et al, 2012), it is not clear exactly how these responses do occur.
334 Our results are consistent with plants using a chemical 'substrate volume-sensing signal' (SVS) to
335 detect their substrate volume early in their lifetime. Our experiments show that dilution of the SVS
336 can occur within the substrate volume even if roots cannot physically access that volume (Figure 4
337 and Figure 5), and imply that the signal is relatively mobile in water, but also in soil. After transfer to
338 a large substrate volume, the inhibitory effect of being grown in a small volume is rapidly alleviated
339 (Figure 4), even if roots cannot grow into the additional substrate volume. This can only be consistent
340 with the rapid dilution of a chemical signal within the substrate volume. We propose that this signal
341 inhibits shoot growth unless diluted into larger volume of substrate; as plants grow, the concentration
342 of SVS increases, gradually inhibiting the shoot growth of plants (Figure 7). Semchenko et al (2007)
343 previously proposed that root exudates have a self-inhibitory effect on root growth. However, our
344 data indicate that the earliest growth responses to volume restriction only occur in the shoot, not the
345 root (Figure 3). Our SVS model is thus qualitatively different to that proposed by Semchenko et al
346 (2007).

347

348 Root density sensing drives later volume responses

349 It is notable that in cases where plants have a substrate volume *x* and a rooting volume *y*, their shoot
350 growth is considerably larger than plants grown in a substrate volume of *y*. However, it is also clear
351 that these plants do not grow as large as plants with a rooting volume of *x*. This suggests that in
352 addition to the concentration of SVS within the substrate, the absolute size of the root system also
353 constrains shoot growth. It was very noticeable in the hydroponic experiment that root system growth
354 was rapidly inhibited in the restrained plants (i.e. there was not only a lower rooting volume, but a
355 fewer roots), and their shoot growth did not accelerate, unlike control plants (Figure 5). Similarly, the
356 maximum level of tillering in the soil transfer experiment was lower for treatment E than for treatments

357 C and D, suggesting that the limited rooting volume eventually inhibited root growth, with a knock-
358 on effect on shoot growth (Figure 4). In the clear pot experiment, we saw that early changes in shoot
359 growth occurred despite continuing root growth, while later changes in shoot growth occurred when
360 root growth stopped or slowed (Figure 3). We thus propose that volume restriction has two phases.
361 There is an early phase in which the concentration of SVS is used to match the rate of shoot growth
362 to the maximum substrate volume, irrespective of rooting volume and the current growth of the root
363 system (Figure 7). Then, in a later phase, the density of roots is used to match the rate of root growth
364 to the maximum rooting volume (as distinct from substrate volume), with further, indirect effects on
365 shoot growth (Figure 7). Thus, when the roots have 'filled' the available rooting volume, root growth
366 is strongly inhibited, imposing a harder limit on shoot growth. The identity of the 'root density-sensing
367 signal' (RDS) for this second phase is also unclear. While this signal could be mechanical, we note
368 that this second signal matches the concept of self-inhibitory signal for root growth proposed by
369 Semchenko et al (2007). Their results suggested this is an organic root exudate, and it thus seems
370 likely that the RDS signal is also chemical rather than physical in nature. However, given that the
371 RDS cannot be effectively diluted in those cases where substrate volume exceeds rooting volume,
372 this suggest the RDS is a much less mobile signal, and therefore probably a much higher molecular
373 weight molecule than SVS.
374

375
Volume sensing and neighbour detection

376 If plants use chemical signals to detect substrate volume, they may also be able to detect each other
377 through the same system. There is certainly abundant evidence that plants can detect their
378 neighbours through both shoot and root systems (Huber et al, 2020; Ninkovic et al, 2020; Wang et
379 al, 2020), but the relative contribution of root and shoot-based detection is unclear, as is the
380 mechanism of root-based detection (Wang et al, 2020). Our data support the importance of root-
381 based signalling in neighbour detection, and we show that the majority of the effects of neighbour
382 detection on shoot growth are mediated through the root system. Since responses to increasing
383 neighbour density can be diminished by increasing substrate volume (and vice versa) (Figure 6), our
384 results suggest that the effects of substrate volume and neighbouring plants are to some extent

385 interchangeable. The effect of crowding is – at least in part – not a response to the presence of
386 neighbouring plants *per se*, and the effect of substrate volume is not purely a response to the size
387 of the container *per se*. Rather, both effects are likely a response to the increasing concentration of
388 SVS (early) and RDS (later) in the shared substrate. This is consistent with a range of previous work
389 that has shown the competition between the root systems of neighbouring plants in part is a density-
390 and nutrient-dependent effect, rather than solely a response to the presence of neighbours p*er se*
391 (Tollenaar & Wu, 1999; Tollenaar et al., 2006; Schenk et al, 2006; Nord et al, 2011; Yan et al., 2017).
392
393 The results from the soil volume/plant density experiment are also consistent with the proposed two-
394 phase mechanism for neighbour detection. It is notable that 4/pot 500ml plants initially added tillers
395 rapidly, with similar kinetics to 1/pot 500ml plants (Figure 6B). Conversely, 1/pot 100ml plants, tillered
396 more slowly from the start (Figure 6B), despite the soil volume per plant being similar for 4/pot 500ml
397 and 1/pot 100ml plants. This suggests that in the large substrate volume, the local level of SVS
398 around each plants roots is diluted more rapidly, even though multiple plants are contributing to the
399 exudation of SVS. However, tillering became inhibited in the 4/pot 500ml treatment by week 5, while
400 1/pot 500ml and 1/pot 100ml plants continued to add tillers until at least week 8 (Figure 6B). While
401 the non-crowded plants have a more linear response to increasing SVS concentration, the 4/pot
402 500ml crowded plants appear to have a hysteric response to SVS, in which they avoid inhibition by
403 SVS early on because of local dilution, but then as SVS levels build up globally due to the presence
404 of multiple plants, they suddenly flip into a completely inhibited state. This difference in the dynamic
405 response of single and crowded plants to the SVS signal doubtlessly reflects the physical/chemical
406 properties of the signal and its dispersal in soil, but these cannot be extrapolated here. The resulting
407 temporal delay in responding to neighbouring plants means that early shoot growth is greater than
408 expected in the crowded plants compared to single plants in a similar volume (Figure 6C), and this
409 improved establishment likely carries over into final biomass.
410
411

412 Density sensing allows pro-active modulation of shoot growth

413 Gardeners everywhere are familiar with concept of plants becoming 'pot-bound'. The restrictive effect
414 of growing plants in small pots has long been recognised, but the mechanisms underlying this
415 phenomenon has remained poorly characterised and understood (Poorter et al., 2012). We show
416 that substrate volume does not impose a static, hard limitation on growth; plants do not simply grow
417 unperturbed until they fill their pot and can grow no more. Rather, we show that plants pro-actively
418 match their growth to the substrate volume, beginning from very early in the life cycle, allowing them
419 to complete their life-cycle without any obvious stress. The adaptive value of the SVS and RDS
420 systems likely relates to 'predicting' the future supply of nutrients and water. The nutrient
421 concentration and the water potential of the soil provide excellent information about *current* resource
422 availability to the plant, but cannot be used to predict future resource availability. However, if a plant
423 can sense it is rooted in a limited volume, this provides information that life-time nutrient availability
424 will likely be limited, as will the maximum amount of water that can be extracted per unit time. The
425 presence of neighbouring plants similarly indicates that both nutrients and water may become limited
426 in the future, even if they are currently abundant. The sensing of substrate volume thus acts as proxy
427 for future resource availability, and allows plants to pro-actively restrict their shoot growth in
428 response. This in turn allows plants to precisely 'plan out' and complete their life-cycle almost
429 irrespective of the available resources.

430

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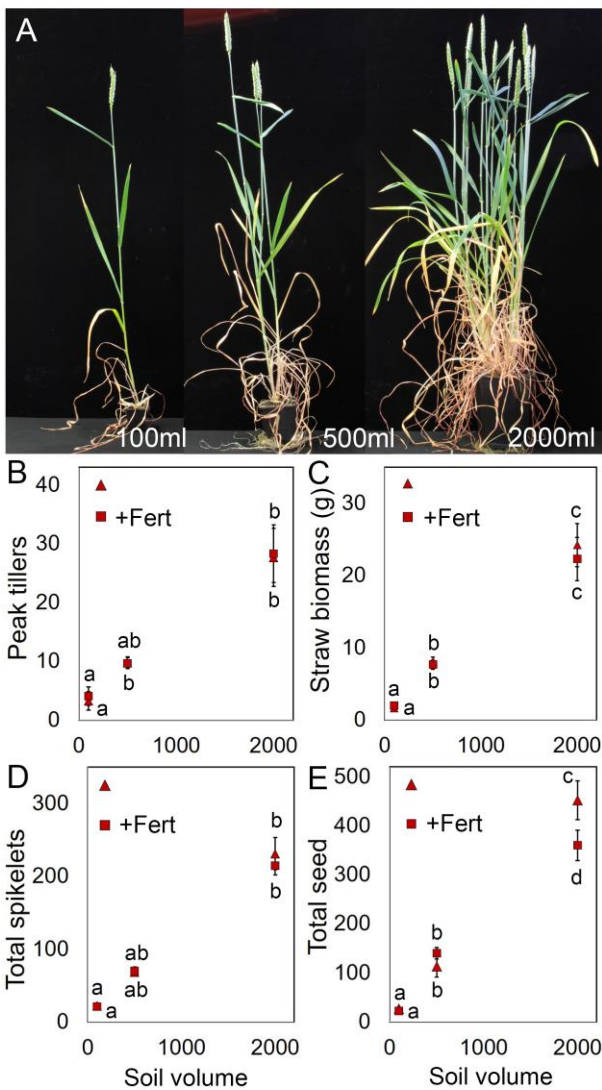
431 FIGURE LEGENDS



432

433 **Figure 1: Soil volume directly influences plant growth independently of nutrient**

434 **levels**

435 **A)** Final plant size in spring wheat plants grown in 100, 500 and 2000ml of soil (photos are to scale).

436 **B-E)** Graphs showing the relationship between soil volume and mean peak tiller number (B), straw

437 biomass (C) mean total spikelets (D) and mean total seed (E) in spring wheat (Mulika) in 100, 500

438 and 2000ml of soil, without supplemental fertiliser (closed triangles) or with additional fertiliser ('Fert')

439 (closed squares). Error bars indicate s.e.m, n=6-12. Data points with the same letter are not

440 statistically different to each other; Kruskal-Wallis (C) or ANOVA + Tukey HSD (C-E).

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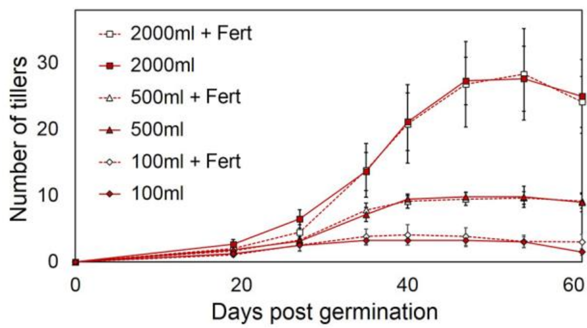

443

444 **Figure 2: Plants respond to soil volume early in the life-cycle**

445 **A)** Graph showing mean tiller number in the 63 days after germination in spring wheat (Mulika) in

446 100ml (diamond markers), 500ml (triangle markers) and 2000ml (square markers) of soil, without

447 supplemental fertiliser (closed markers, solid lines) or with supplemental fertiliser (open markers,

448 dashed lines). Error bars indicate s.e.m, n=6-12.

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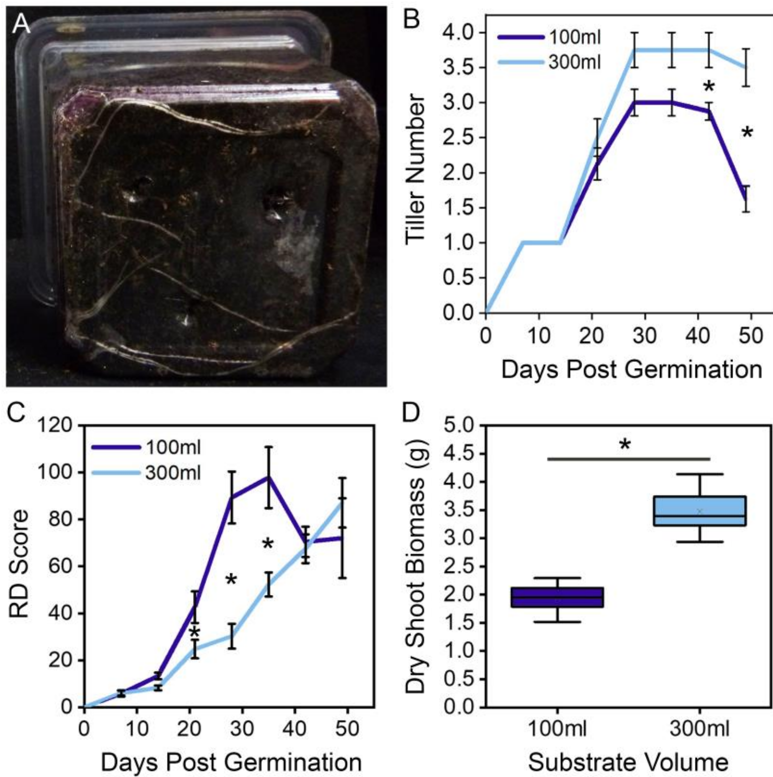

458 **Figure 3: Early shoot growth is not correlated with mechanical stimulus or root**

459 **growth**

460 **A)** Root development in spring wheat grown in clear sided 300ml pots, 1 week after germination.

461 Scale bar= 10cm

462 **B)** Graph showing mean tiller number in the 50 days after germination in spring wheat (Mulika) grown

463 in 100ml (dark blue) and 300ml (light blue) clear-sided pots containing soil. Error bars indicate s.e.m,

464 n=8 for each pot size. Asterisks indicate significant differences between treatments (Mann-Whitney

465 U; $p<0.05$).

466 **C)** Graph showing visible root density of wheat plants grown in 100ml (dark blue) and 300ml (light

467 blue) pots in the 50 days post germination, measured as 'root density score' (see Methods). Error

468 bars indicate s.e.m, n=5-8 for each pot size. Asterisks indicate significant differences between

469 treatments at each time point (Day 21 and 28 t-test; $p<0.05$, Day 35 Mann-Whitney U test; $p<0.05$).

470 **D)** Box plot showing final shoot biomass in wheat plants grown in 100ml (dark blue) and 300ml (light

471 blue) pots for 8 weeks. The box represents the interquartile range, whiskers show the maximum and

472 minimum values, the midline represents the median, the x represents the mean. Asterisks indicate

473 significant difference from 100ml treatments (t-test; $p<0.05$).

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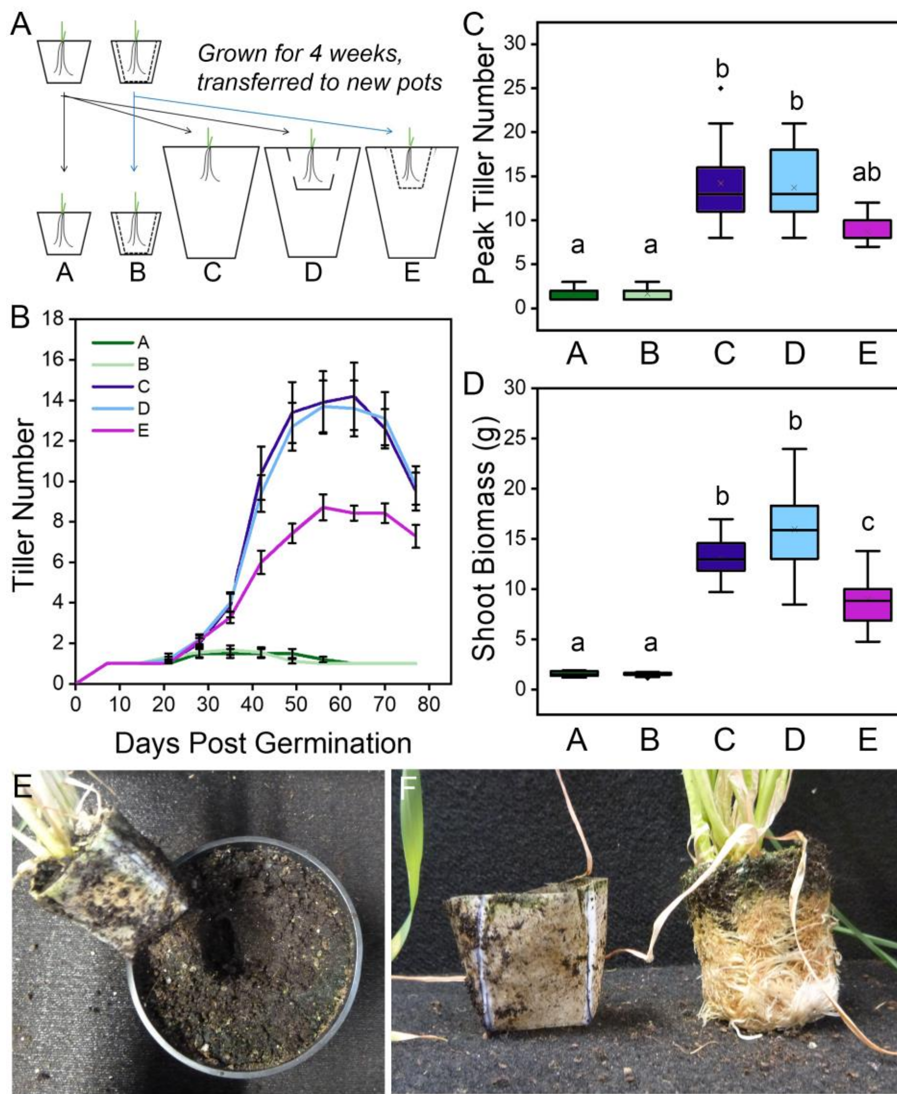

475 **Figure 4: A mobile signal drives substrate volume sensing**

476 **A)** Cartoon showing set up for experiment.

477 **B)** Graph showing mean tiller number in the 77 days after germination in spring wheat (Mulika) grown
478 in the different treatments shown in **A)**. Error bars indicate s.e.m, n=7-10 for each treatment.

479 **C**) Box plot showing peak tiller number for each treatment. The box represents the interquartile
480 range, whiskers show the maximum and minimum values, the midline represents the median, the x
481 represents the mean and diamonds represent outliers. n=7-10 for each treatment. Boxes with the
482 same letter are not significantly different (Kruskal Wallis Pairwise Comparisons with Bonferroni
483 Correction; $p<0.05$). Treatments A and B are the dark and light lines at the bottom of the graph; C
484 and D are the dark and light lines at the top of the graph, E is the mid-toned line in the middle of the
485 graph.

486 **D)** Box plot showing final shoot biomass in the different treatments shown in **A)**. The box represents
487 the interquartile range, whiskers show the maximum and minimum values, the midline represents
488 the median, the x represents the mean. n=7-10 for each treatment. Boxes with the same letter are
489 not significantly different (ANOVA + Tukey HSD $p<0.05$).
490 **E, F**) Photograph showing the lack of root growth outside the mesh pot, and the intense rooting ball
491 formed inside the mesh pots in treatment E plants.
492

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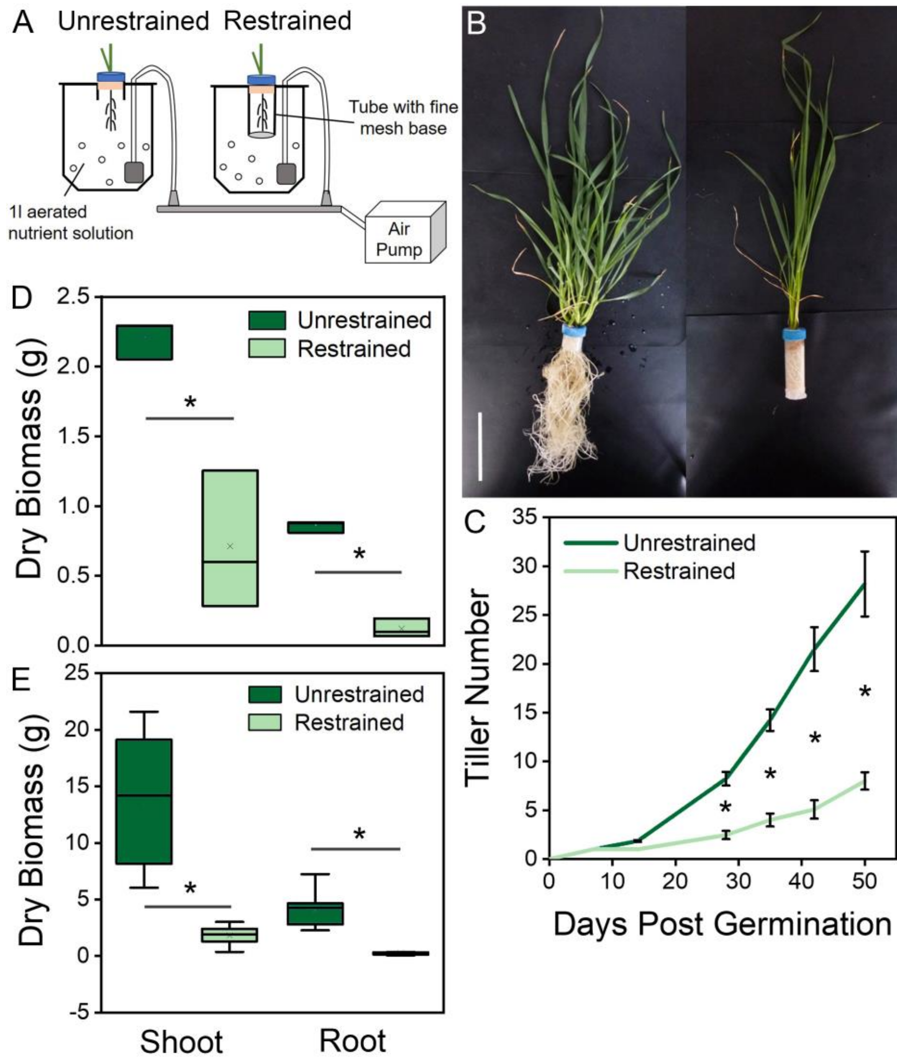

494

495 **Figure 5: A mobile signal drives substrate volume sensing**

496 **A)** Cartoon showing set up for experiment.

497 **B)** Photo of 6 week-old plants grown in this experiment; unrestrained (left) and restrained (right).

498 **C)** Graph showing mean tiller number in the 52 days after germination in spring wheat (Mulika) grown

499 in the unrestrained (dark green) versus restrained (light green) treatments shown in **A).** Error bars

500 indicate s.e.m, n=10 for each treatment. Asterisks indicate significant differences between

501 treatments (Day 28, 35, 42 Mann-Whitney U test; $p<0.05$, Day 52 t-test; $p<0.05$).

502 **D)** Box plot showing shoot and root biomasses at 42 days post germination in the unrestrained (dark

503 green) versus restrained (light green) treatments shown in **A).** The box represents the interquartile

504 range, whiskers show the maximum and minimum values, the midline represents the median, the x

505 represents the mean. n=3 for each treatment. Asterisks indicate significant differences relative to

506 unrestrained plants at the same timepoint (t-test; $p<0.05$).

507 **E)** Box plots showing shoot and root biomasses at 52 days post germination in the unrestrained (dark

508 green) versus restrained (light green) treatments shown in **A).** The box represents the interquartile

509 range, whiskers show the maximum and minimum values, the midline represents the median, the x

510 represents the mean. n=8-10 for each treatment. Asterisks indicate significant differences relative to

511 unrestrained plants at the same timepoint (t-test; $p<0.05$).

512

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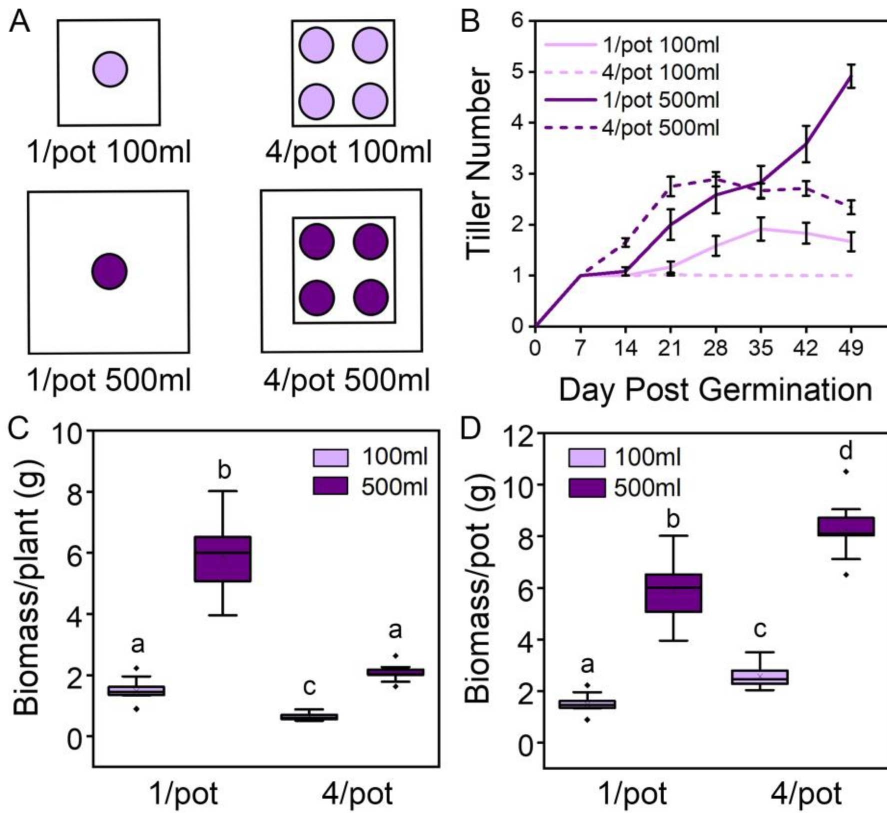

514

515 **Figure 6: Neighbour density and soil volume interchangeably inhibit shoot**

516 **growth**

517 **A)** Cartoon showing set up for experiment. Light purple dots represent spring wheat (Mulika) plants

518 grown in 100ml pots, dark purple dots represent wheat plants grown in 500ml pots. The inner square

519 for 4/pot 500ml represents a template used for the same surface area as the 100ml plants.

520 **B)** Graph showing mean tiller number between in the 49 days post germination in spring wheat

521 (Mulika) grown on either 100ml (light purple) or 500ml (dark purple) of soil, at a rate of 1/pot (solid

522 lines) and 4/pot (dashed lines). Error bars indicate s.e.m, n=11-12.

523 **C,D)** Box plots showing total shoot biomass per pot (C) and per plant (D) in spring wheat (Mulika)

524 grown in 100ml (light purple, left in each pair of boxes) or 500ml (dark purple, right in each pair of

525 boxes) of soil, or a rate of 1/pot and 4/pot. Box represents interquartile range, and midline indicates

526 the median. Diamonds above and below the whiskers indicate outliers. Whiskers indicate maximum

527 and minimum. Bars with the same letter are not statistically different from each other (One-way

528 ANOVA + Tukey HSD, n=11-12 pots, $p<0.05$).

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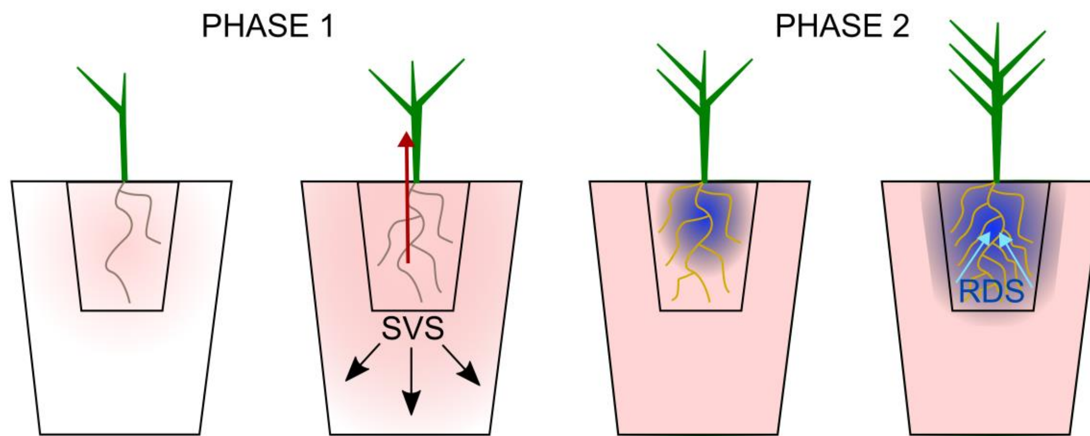

530

531 **Figure 7: A model for substrate volume effects on shoot growth**

532 We propose there are two distinct phases in which exudate-based signalling in the root system
533 influences shoot growth. In phase 1, a highly mobile 'substrate volume-sensing signal' (SVS) is
534 produced by the roots (pink shading), and diluted within the substrate volume (outer pot), even if the
535 potential rooting volume is smaller (inner pot) (left image). As levels of SVS increase, the plant
536 perceives the limits to its substrate volume (left centre image), and root-to-shoot signalling (red
537 arrow) downregulates shoot growth to match the plants to their maximum possible substrate volume.
538 Later in development, in phase 2, the levels of a less mobile 'root density-sensing signal' (RDS)
539 produced by the roots (blue shading) start to rise, as root density within the rooting volume becomes
540 appreciable (right centre image). Once root density reaches a critical level (right image), the RDS
541 inhibits root growth (blue arrows), with knock-on, allometric effects on shoot growth. Although RDS
542 is somewhat mobile, it cannot be diluted within the whole substrate volume, and so its distribution
543 largely reflects the size of the rooting volume, rather than the substrate volume.

544

Variety	Volume (ml)	Fert	Ear		Spikelet	Seed	
			Number	Mass (g)	Number	Number	Mass (g)
Mulika	100	-	1.00 ±0.00	1.08 ±0.11	22.50 ±0.50	26.54 ±2.93	0.86 ±0.12
	100	+	0.91 ±0.08	0.81 ±0.10	21.27 ±0.37	22.00 ±2.11	0.55 ±0.09
	500	-	2.83 ±0.15	6.88 ±0.41	68.33 ±3.90	113.17 ±21.43	5.23 ±0.34
	500	+	3.00 ±0.23	6.67 ±0.51	70.17 ±5.47	140.17 ±11.68	5.09 ±0.47
	2000	-	9.33 ±0.73	21.55 ±1.72	231.33 ±21.86	452.17 ±39.23	14.90 ±1.17
	2000	+	9.33 ±0.56	17.66 ±2.36	214.83 ±13.02	360.17 ±30.99	14.40 ±2.52

545

546 **Supplementary Table 1: Soil volume directly influences wheat reproductive**
547 **architecture**

548 Table showing reproductive parameters in spring wheat (Mulika) grown in 100, 500 and 2000ml pots,
549 with or without additional fertiliser. Data are means ± s.e.m., n=6-12.