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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

shoot meristem activity, leaf size and shape, leaf senescence shoot branch number and stem elongation (Wheeldon & Bennett, 2020). This allows plants to match the overall rate of shoot growth to the availability of mineral nutrients, so that growth can be sustained in the long term.

Plants can also use a wide range of non-nutritional information to guide their growth. This non-nutritional information is particularly useful where it allows plants to 'predict' future resource scarcity, even when current resources are abundant. Again, this allows plants to pro-actively limit growth to avoid resource limitations (Walker & Bennett, 2018). The light reflected by neighbouring plants provides an excellent example of such non-nutritional information. Light reflected from leaves has a much lower ratio of red:far red wavelengths than unreflected sunlight, due to absorbance of red light for photosynthesis (Ballaré & Pierik, 2017). Thus, plants can use the enrichment of far red light to detect the presence of neighbouring plants, and to predict future competition for light (Roig-Villanova & Martínez-García, 2016). In response, they can modulate their growth, restricting branching and promoting stem elongation to outgrow the competing plant. This response does not require active shading, and plants can detect neighbours over some distance via light cues (Roig-Villanova & Martínez-García, 2016). Indeed a 'spike' of far red light is sufficient to induce neighbour detection responses without any difference in the intensity of photosynthetically active wavelengths (Xie et al., 2020). Thus, anticipation of future light limitation is sufficient to modulate growth, in the absence of any underlying resource limitations.

Besides the availability of mineral nutrients, there are other physical and biological components of the rhizosphere that plants could use to predict the likelihood of resource availability in the future. Limiting the substrate volume available to a plant (usually achieved with a plant pot) has a very strong effect on shoot growth (McConaughey & Bazazz, 1991; Hess & de Kroon, 2006; Poorter at al, 2012). This 'volume restriction' phenomenon has previously been investigated across a range of species including bean, cotton and tomato (Carmi & Heuer, 1981; Gurevitch et al., 1990; Bar-Tal et al., 1995; Xu et al., 2001; Yong et al., 2010). These volume restriction effects are also not caused by water deficit (Krizek et al., 1985; Ismail & Davies, 1998) and indeed, still occur in hydroponic conditions (Ternesi et al., 1994; Bar-Tal et al., 1995; Shi et al., 2008); nor are they correlated with

reduced photosynthetic capacity (Carmi & Heuer, 1981; Kharkina et al., 1999; Shi et al., 2008; Yong et al., 2010). Furthermore, although it difficult to experimentally separate the effects of substrate volume and nutrient availability, it is generally accepted that volume restriction is not caused by the availability of nutrients in the substrate (Hess & de Kroon, 2006), a view supported by a meta-analysis of 65 different studies (Poorter et al., 2012). Most notably, using a hydroponic system, Bar-Tal et al (1995) showed that the growth of tomato plants was proportional to the size of the permeable bag they were grown inside, even though they had access to same total volume of nutrient solution. Various factors have been proposed to explain volume restriction effects, including mechanical interactions with pot walls, or the increased heat that occurs in small pots (Poorter et al., 2012), while Falik et al (2005) and Semchenko et al (2007) suggested that self-inhibitory root exudates might account for the changes in root growth that occur in the presence of obstacles and or in limited soil volumes. Currently however, the mechanism by which substrate volume effects occur remains unclear.

We hypothesise that plants use substrate volume as a form of information to dynamically regulate their growth. That is to say, we hypothesise that plants do not simply grow maximally until they fill their pot, but that they respond to substrate volume early in development to match their growth to the size of the pot. In this study, we tested this idea in hexaploid bread wheat, and attempted to understand how these soil volume effects arise. We show that soil volume effects on shoot growth are indeed proactive, and that they are unlikely to arise from interactions with pot walls. Rather, our data support a model in which plants release and detect two different signals into the rhizosphere; by detecting the increasing concentration of these signals during different phases of growth, they are able to 'measure' substrate volume. Moreover, we show that detection of these signal may also strongly contribute to the detection of neighbouring plants, and allow plants to integrate both soil volume and neighbour density effects into a coherent response.

MATERIALS & METHODS

Plant growth conditions and materials

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

Phenotypic assessments

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

Root density quantification

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

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

Experimental design

Figure 1 and Figure 2

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

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

Figure 3

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

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

RESULTS

Shoot growth responds to soil volume independently of nutrient level

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

Shoot responses to soil volume are pro-active

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

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

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

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

Volume responses do not require volume occupation

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

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

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

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

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

Shoot responses to soil volume and crowding are partly interchangeable

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

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

DISCUSSION

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

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

Root density sensing drives later volume responses

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

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

Volume sensing and neighbour detection

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

interchangeable. The effect of crowding is – at least in part – not a response to the presence of neighbouring plants *per se*, and the effect of substrate volume is not purely a response to the size of the container *per se*. Rather, both effects are likely a response to the increasing concentration of SVS (early) and RDS (later) in the shared substrate. This is consistent with a range of previous work that has shown the competition between the root systems of neighbouring plants in part is a density- and nutrient-dependent effect, rather than solely a response to the presence of neighbours p*er se* (Tollenaar & Wu, 1999; Tollenaar et al., 2006; Schenk et al, 2006; Nord et al, 2011; Yan et al., 2017).

The results from the soil volume/plant density experiment are also consistent with the proposed two-phase mechanism for neighbour detection. It is notable that 4/pot 500ml plants initially added tillers rapidly, with similar kinetics to 1/pot 500ml plants (Figure 6B). Conversely, 1/pot 100ml plants, tillered more slowly from the start (Figure 6B), despite the soil volume per plant being similar for 4/pot 500ml and 1/pot 100ml plants. This suggests that in the large substrate volume, the local level of SVS around each plants roots is diluted more rapidly, even though multiple plants are contributing to the exudation of SVS. However, tillering became inhibited in the 4/pot 500ml treatment by week 5, while 1/pot 500ml and 1/pot 100ml plants continued to add tillers until at least week 8 (Figure 6B). While the non-crowded plants have a more linear response to increasing SVS concentration, the 4/pot 500ml crowded plants appear to have a hysteric response to SVS, in which they avoid inhibition by SVS early on because of local dilution, but then as SVS levels build up globally due to the presence of multiple plants, they suddenly flip into a completely inhibited state. This difference in the dynamic response of single and crowded plants to the SVS signal doubtlessly reflects the physical/chemical properties of the signal and its dispersal in soil, but these cannot be extrapolated here. The resulting temporal delay in responding to neighbouring plants means that early shoot growth is greater than expected in the crowded plants compared to single plants in a similar volume (Figure 6C), and this improved establishment likely carries over into final biomass.

Density sensing allows pro-active modulation of shoot growth

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

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FIGURE LEGENDS

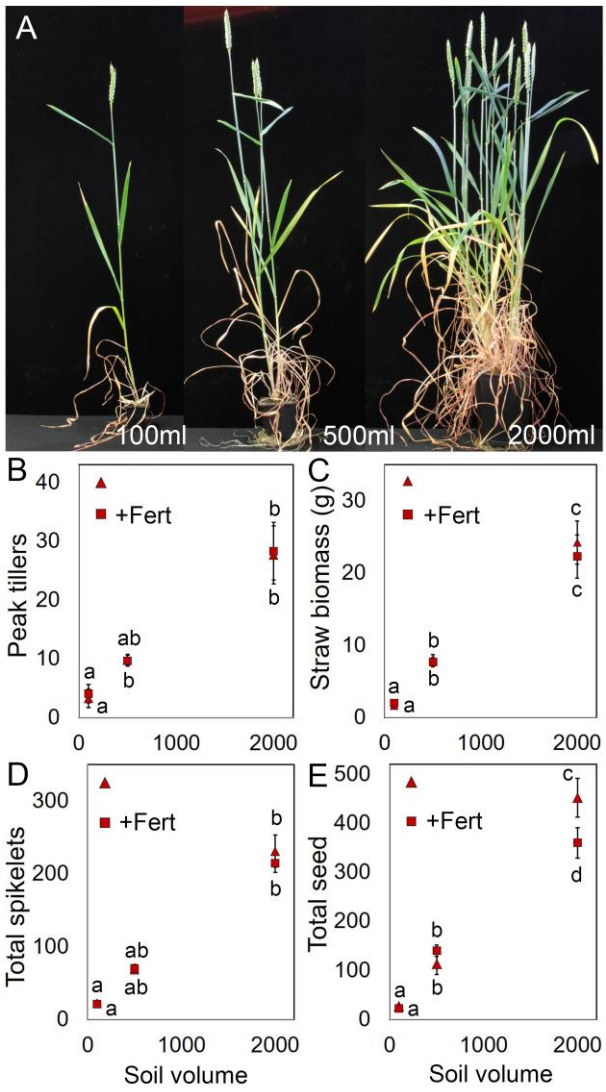

Figure 1: Soil volume directly influences plant growth independently of nutrient levels

A) Final plant size in spring wheat plants grown in 100, 500 and 2000ml of soil (photos are to scale). **B-E)** Graphs showing the relationship between soil volume and mean peak tiller number (B), straw biomass (C) mean total spikelets (D) and mean total seed (E) in spring wheat (Mulika) in 100, 500 and 2000ml of soil, without supplemental fertiliser (closed triangles) or with additional fertiliser ('Fert') (closed squares). Error bars indicate s.e.m, n=6-12. Data points with the same letter are not statistically different to each other; Kruskal-Wallis (C) or ANOVA + Tukey HSD (C-E).

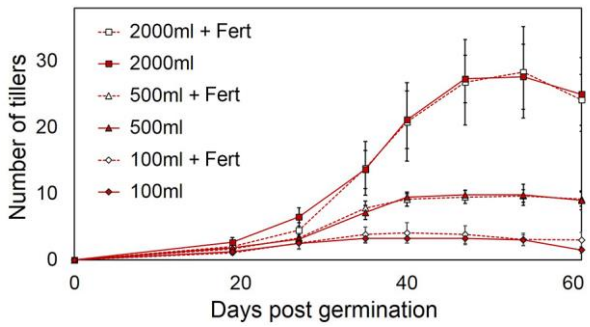

Figure 2: Plants respond to soil volume early in the life-cycle

A) Graph showing mean tiller number in the 63 days after germination in spring wheat (Mulika) in 100ml (diamond markers), 500ml (triangle markers) and 2000ml (square markers) of soil, without supplemental fertiliser (closed markers, solid lines) or with supplemental fertiliser (open markers, dashed lines). Error bars indicate s.e.m, n=6-12.

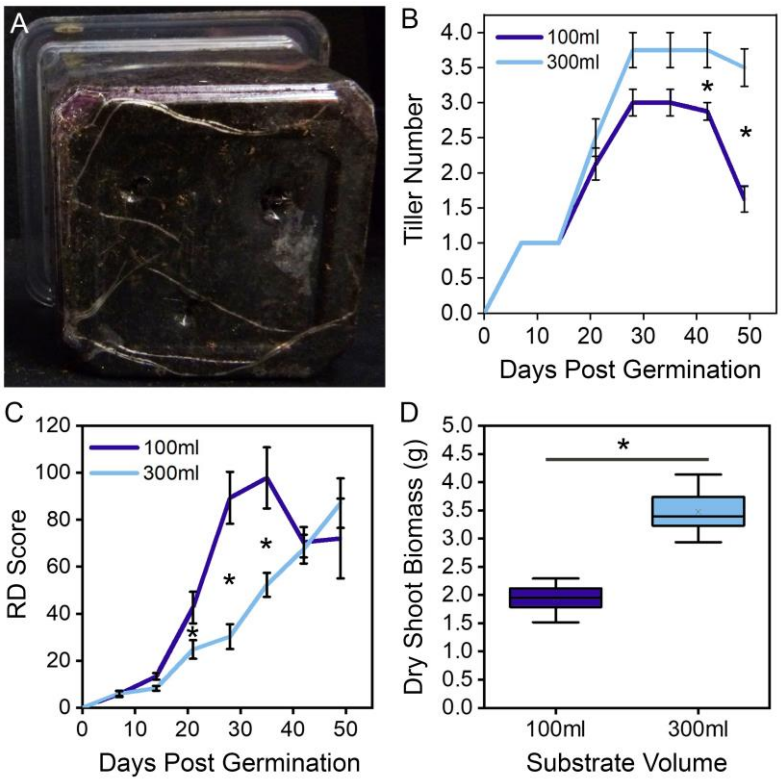

Figure 3: Early shoot growth is not correlated with mechanical stimulus or root growth

A) Root development in spring wheat grown in clear sided 300ml pots, 1 week after germination. Scale bar= 10cm

B) Graph showing mean tiller number in the 50 days after germination in spring wheat (Mulika) grown in 100ml (dark blue) and 300ml (light blue) clear-sided pots containing soil. Error bars indicate s.e.m, n=8 for each pot size. Asterisks indicate significant differences between treatments (Mann-Whitney U; $p<0.05$).

C) Graph showing visible root density of wheat plants grown in 100ml (dark blue) and 300ml (light blue) pots in the 50 days post germination, measured as 'root density score' (see Methods). Error bars indicate s.e.m, n=5-8 for each pot size. Asterisks indicate significant differences between treatments at each time point (Day 21 and 28 t-test; $p<0.05$, Day 35 Mann-Whitney U test; $p<0.05$).

D) Box plot showing final shoot biomass in wheat plants grown in 100ml (dark blue) and 300ml (light blue) pots for 8 weeks. The box represents the interquartile range, whiskers show the maximum and minimum values, the midline represents the median, the x represents the mean. Asterisks indicate significant difference from 100ml treatments (t-test; $p<0.05$).

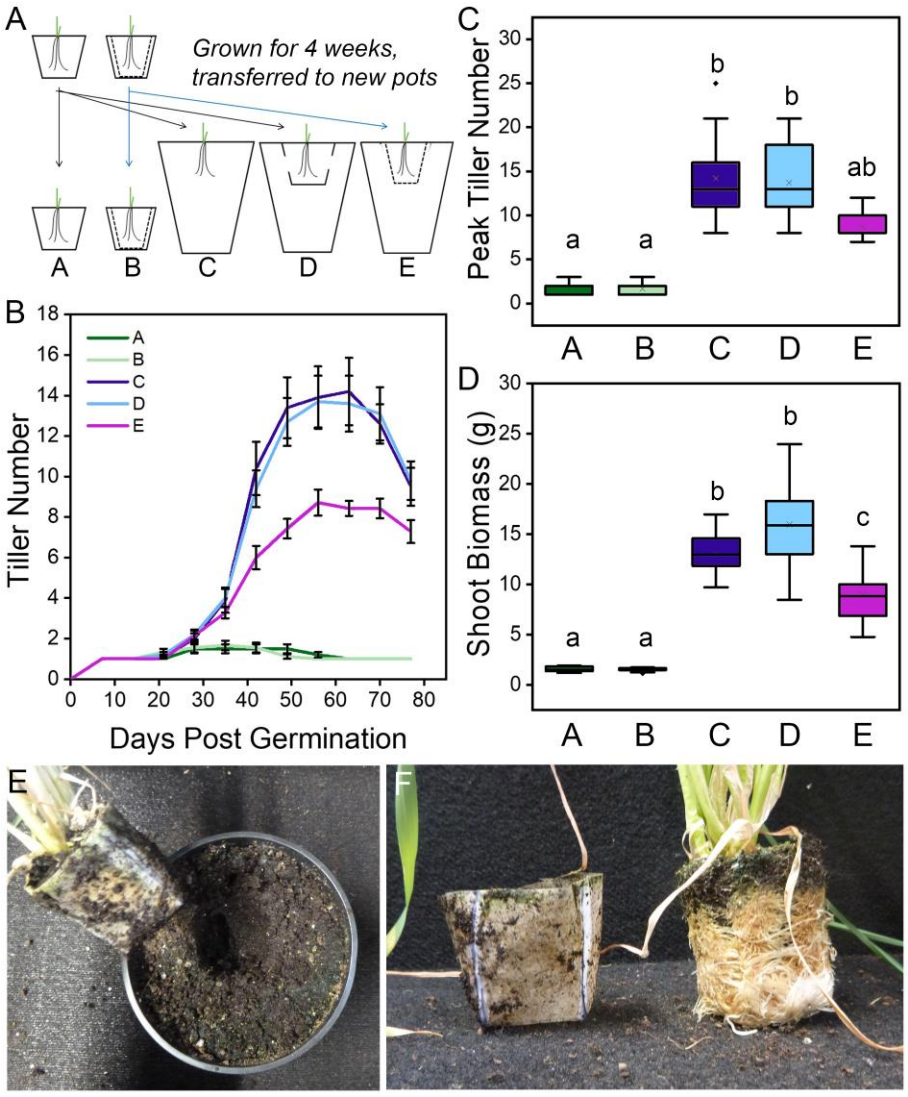

Figure 4: A mobile signal drives substrate volume sensing

A) Cartoon showing set up for experiment.

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

C) Box plot showing peak tiller number for each treatment. The box represents the interquartile range, whiskers show the maximum and minimum values, the midline represents the median, the x represents the mean and diamonds represent outliers. n=7-10 for each treatment. Boxes with the same letter are not significantly different (Kruskal Wallis Pairwise Comparisons with Bonferroni Correction; $p<0.05$). Treatments A and B are the dark and light lines at the bottom of the graph; C 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 graph.

D) Box plot showing final shoot biomass in the different treatments shown in **A)**. The box represents the interquartile range, whiskers show the maximum and minimum values, the midline represents the median, the x represents the mean. n=7-10 for each treatment. Boxes with the same letter are not significantly different (ANOVA + Tukey HSD $p<0.05$).

E, F) Photograph showing the lack of root growth outside the mesh pot, and the intense rooting ball formed inside the mesh pots in treatment E plants.

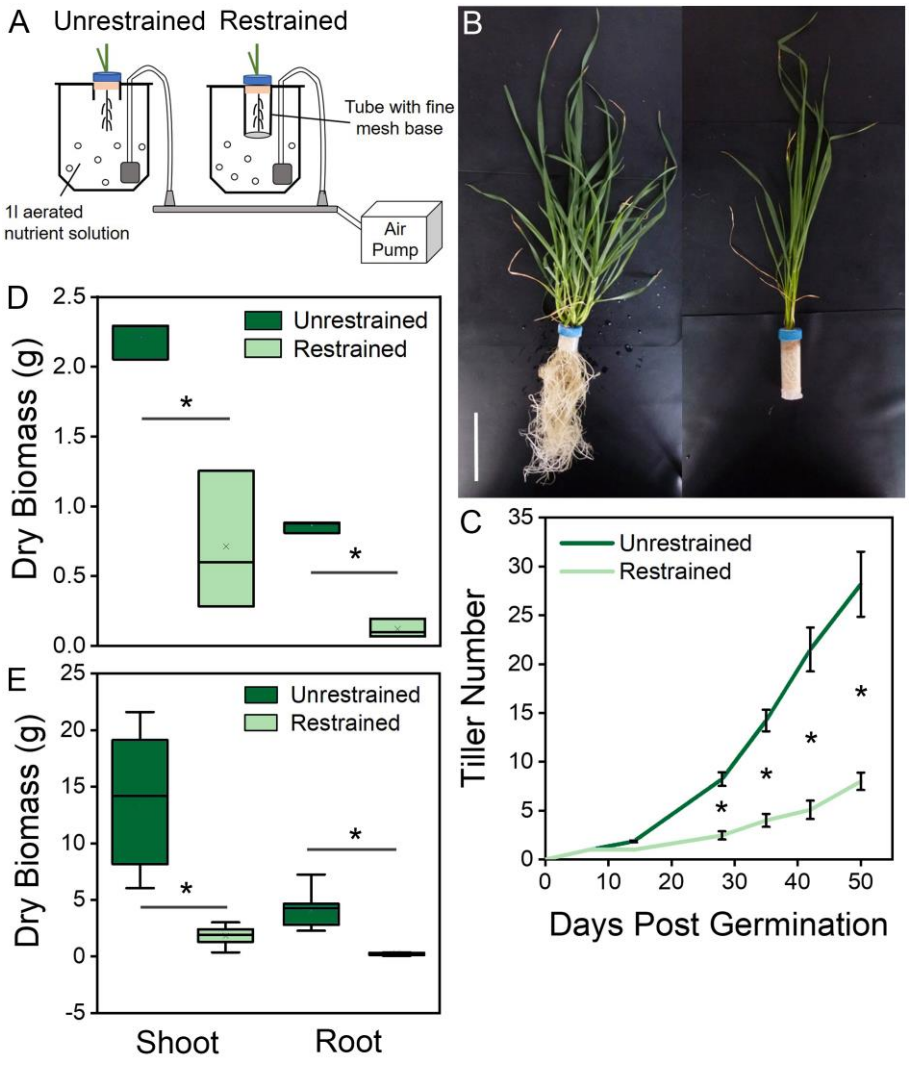

Figure 5: A mobile signal drives substrate volume sensing

A) Cartoon showing set up for experiment.

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

C) Graph showing mean tiller number in the 52 days after germination in spring wheat (Mulika) grown in the unrestrained (dark green) versus restrained (light green) treatments shown in **A)**. Error bars indicate s.e.m, n=10 for each treatment. Asterisks indicate significant differences between treatments (Day 28, 35, 42 Mann-Whitney U test; $p<0.05$, Day 52 t-test; $p<0.05$).

D) Box plot showing shoot and root biomasses at 42 days post germination in the unrestrained (dark green) versus restrained (light green) treatments shown in **A)**. The box represents the interquartile range, whiskers show the maximum and minimum values, the midline represents the median, the x

represents the mean. n=3 for each treatment. Asterisks indicate significant differences relative to unrestrained plants at the same timepoint (t-test; $p<0.05$).

E) Box plots showing shoot and root biomasses at 52 days post germination in the unrestrained (dark green) versus restrained (light green) treatments shown in **A)**. The box represents the interquartile range, whiskers show the maximum and minimum values, the midline represents the median, the x represents the mean. n=8-10 for each treatment. Asterisks indicate significant differences relative to unrestrained plants at the same timepoint (t-test; $p<0.05$).

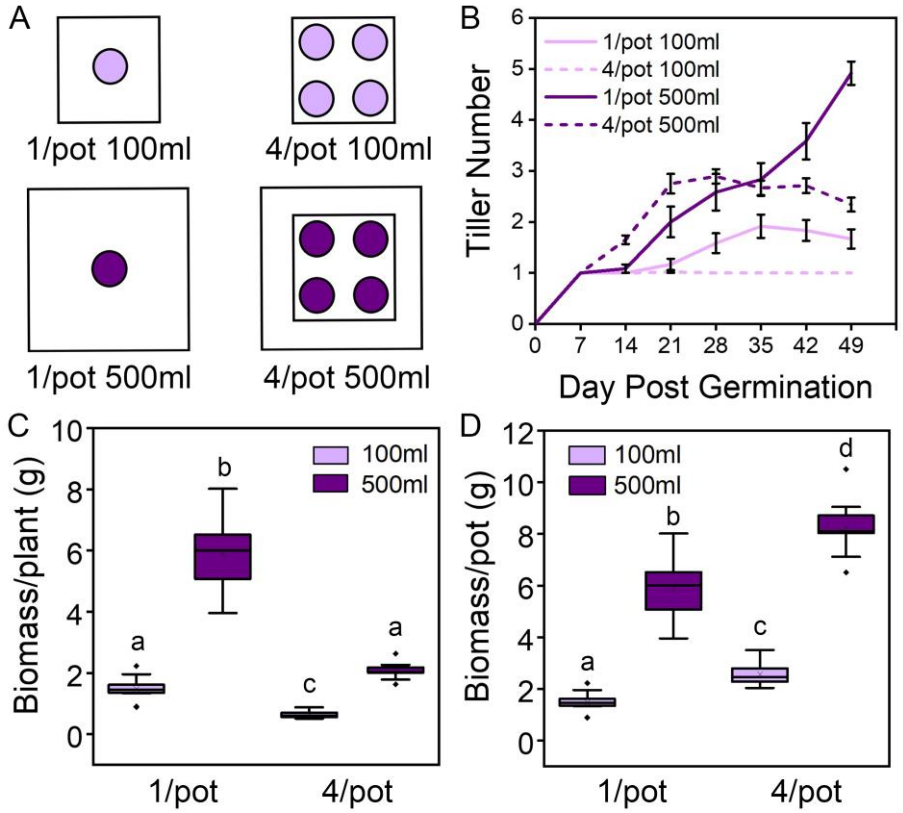

Figure 6: Neighbour density and soil volume interchangeably inhibit shoot growth

A) Cartoon showing set up for experiment. Light purple dots represent spring wheat (Mulika) plants grown in 100ml pots, dark purple dots represent wheat plants grown in 500ml pots. The inner square for 4/pot 500ml represents a template used for the same surface area as the 100ml plants.

B) Graph showing mean tiller number between in the 49 days post germination in spring wheat (Mulika) grown on either 100ml (light purple) or 500ml (dark purple) of soil, at a rate of 1/pot (solid lines) and 4/pot (dashed lines). Error bars indicate s.e.m, n=11-12.

C,D) Box plots showing total shoot biomass per pot (C) and per plant (D) in spring wheat (Mulika) grown in 100ml (light purple, left in each pair of boxes) or 500ml (dark purple, right in each pair of boxes) of soil, or a rate of 1/pot and 4/pot. Box represents interquartile range, and midline indicates the median. Diamonds above and below the whiskers indicate outliers. Whiskers indicate maximum and minimum. Bars with the same letter are not statistically different from each other (One-way ANOVA + Tukey HSD, n=11-12 pots, $p<0.05$).

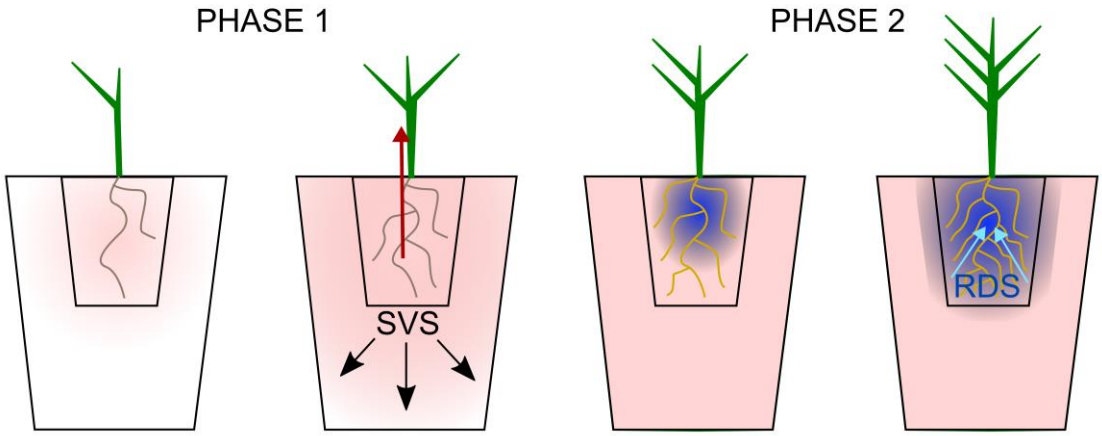

Figure 7: A model for substrate volume effects on shoot growth

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

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

Supplementary Table 1: Soil volume directly influences wheat reproductive architecture

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