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Root Elongation in Agar Gels: Correlation with Agar Concentration and Rheology

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Abstract

Resistance from agar medium affects root growth significantly. Mechanical properties of agar and effects of agar stiffness on root elongation were evaluated through the compression test, modeling, and correlation analysis. The results showed that variation of compression elasticity was not monotonic. The relationship between root growth and agar strength was evaluated by growing Arabidopsis in 1.0−2.0% agar. There were negative correlations between root length and fracture stress and between straightness and Young’s Modulus. In conclusion, change of rheology of agar medium was induced by agar contents, differences in physical properties of agar media modulated root elongation.

Keywords: Agar medium; Mechanical strength; Stiffness; Elasticity; Plant culture; Root elongation

Introduction

The mechanical stimulus can influence plant architecture by triggering organ initiation and causing alterations in the stem or root growth direction [1,2]. Plants have evolved various responsive behaviors to adapt to these stimuli [3-5]. In biomechanical experiments of plants, the mechanical properties of the plant growth medium, such as the stiffness and surface roughness, influence plant architecture [6-8]. Varying amounts of agar in culture medium have been used to regulate the mechanical strength of plant growth [9-11]. The application of high stability and green composite agar as a curing agent in plant culture medium has advanced research in plant development remarkably [8,12].

In recent decades, agar has been mainly used in root cultivation because soil particles bound to roots are difficult to remove without causing injury to roots [13]. Agar concentrations of 0.5%−2.0% (w/v) in half-strength

Murashige and Skoog (MS) medium were commonly used to regulate the stiffness of root growth medium and to investigate the root mechanical response [13,14]. Plant and root culture depend on the properties of the growth medium, especially mechanical stuff. The report about the relationship between agar concentrations and Young's modulus was only for 0.5%−0.7% agar [11]. Although there is an absence of agar medium's physicochemical properties, numerous studies have been conducted concerning the growth behavior of Arabidopsis root, which occurred on agar surface or the semi-solid agar medium [14,15]. When Arabidopsis roots were cultivated on the impenetrable agar surface, which was not parallel to the gravity vector, the roots grow in a waving manner. The waving intensity was correlated with agar concentration [9,16]. Additionally, roots growing on vertical agar surface and inside agar showed a shallow wave [17,18]. Although many papers have been published on Arabidopsis root behavior using agar medium, the rheological characteristics of the agar medium are still unknown in detail, with which we can apply to the quantitative study of the root-gel interaction. Therefore, it is essential to study the mechanical properties of agar in depth. Agar, sucrose, and 1/2 MS medium were blended to form an interpenetrating net-work-like cross-linked complex [19]. With increasing concentration of agar, agar medium gel-forming ability is expected to be influenced, but this has not been studied quantitatively. How does agar concentration modulate medium stiffness? We proposed two different hypotheses to answer the above question: (i) change of mechanical strength of agar medium induced by agar contents; (ii) nonlinear increase of medium stiffness with agar concentrations. To examine the validity of the above hypotheses and evaluate the effect of agar gel resistance on root growth, we quantified the mechanical properties of root culture medium containing a range of agar concentrations through a uniaxial compression test. We observed primary root length, straightness in different agar concentrations. Using physical analysis to explore the interrelation between root elongation and rheology of agar medium, we checked which agar medium can be used for better root growth.

Results and Discussion

Fracture strength

Gelling agent concentrations were varied to study the effects on the fracture strength properties. Loading force-displacement curves were varied with different gelling agent concentrations. The force on each specimen would make a linear increase along with the increment of displacement **(Figure 1).** Fracture stress (calculated from data points in the fracture load) increased with increasing concentration of agar up to 1.0% and did not change very much from 1.0% to 1.3%, and then it began to increase gradually from 1.4% to 2.0%. As the agar concentration (x) increased, value of fracture stress (y) increased logarithmically ($y = 0.0166\ln(x) + 0.0141$). In terms of fracture stress, the maximum value was measured for as high as 1.9%. The lowest agar concentration has the bottom value, suggesting a comparatively more ductile structure in the low concentrations **(Figure 2).**

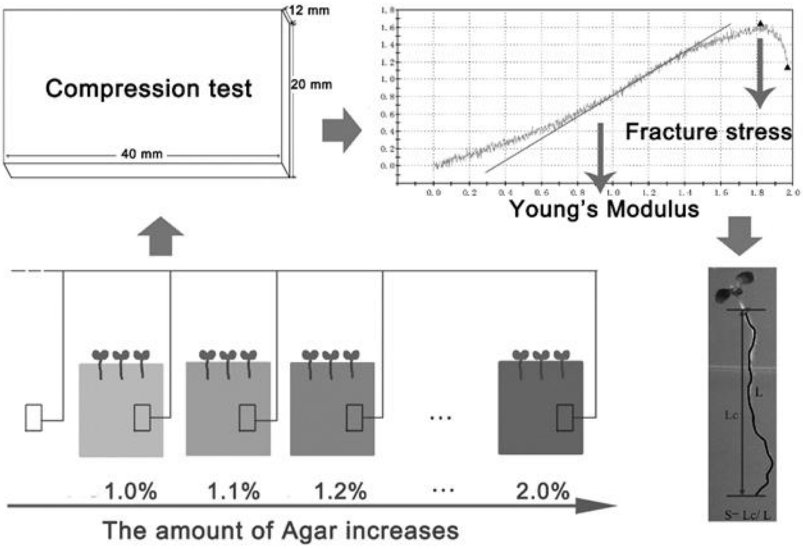

Figure 1: A schematic of studying mechanical properties of 0.5%−2.0% agar and exploring the root response to mechanical stimulation of culture medium.

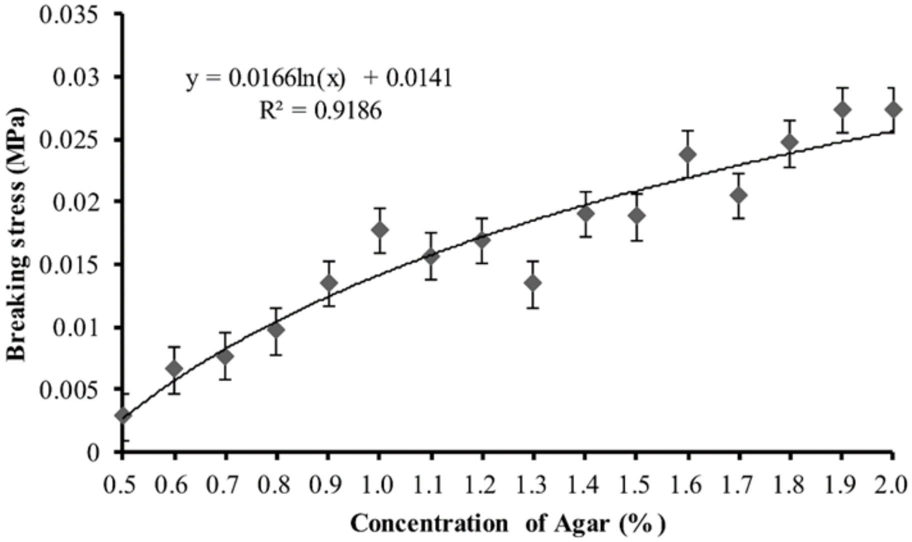

Figure 2: Fracture stress (MPa) of agar media with agar concentration from 0.5%−2.0%. An equation showed the relationship between fracture stress and agar concentration. The model (solid black line) is statistically significant with a p-value <0.001 adjusted R-square value of 90%. Values represent the mean ± SD from three independent experiments.

Young's modulus

It can be seen from the data presented in **Table 1** that, as the percentage of agar in-creased from 0.5% to 1.1%, Young's modulus increased from 0.0124 to 0.0990 MPa. With the same medium composition, agar at 1.1% generally tended to result in a higher stiff-ness or elasticity than others. We observed a relative decrease of young's modulus with the increase of agar concentration from 1.2% to 1.4%, while Young's modulus decreased at high agar concentration from 1.5% to 2.0% **(Table 1).** An analysis of repeated measures design was done using the data provided. The result is that the agar with different concentrations has a distinct influence on the stiffness of the plant culture media with a p-value <0.0001.

Table 1: Young's modulus of 0.5%-2.0% agar media containing 1.5% sucrose and 1/2 MS salts.

The concentration of agar	Young's modulus (MPa)
0.5%	0.0124 ±0.00061
0.6%	0.0290±0.00240
0.7%	0.0278±0.00265
0.8%	0.0365±0.00172
0.9%	0.0526±0.00533
1.0%	0.0650±0.00738
1.1%	0.0990±0.01658
1.2%	0.0576±0.00205
1.3%	0.0686±0.00878
1.4%	0.0204±0.00052
1.5%	0.0413±0.00125
1.6%	0.0747±0.00330
1.7%	0.0973±0.00446
1.8%	0.0435±0.00180
1.9%	0.0439±0.00137
2.0%	0.0449±0.00172

Then further modeling using the spline regression is subsequently applied to the data for agar to establish the relationship between agar concentration level and the stiffness of the culture media **(Figure 3).** It's worth noting that the slop of the modeling curve from 0.5% to 1.1% was small. In other words, stiffness increased slowly when agar concentration increased to 1.1%, and started decreasing gradually when agar concentration ranging from 1.2% to 1.4%. It could be concluded that the mechanical stiffness of the agar medium had a transition point at 1.1%. Generally, a higher agar concentration over 1.2% would not enhance stiffness or compression elasticity for the plant culture medium.

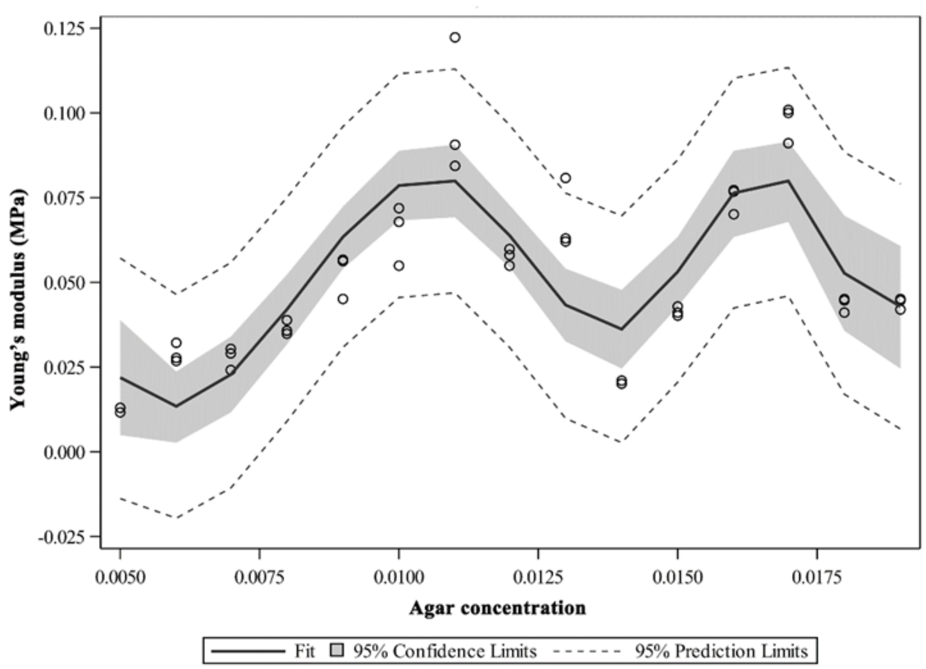

Figure 3: Summary of the model fitting to the Young's modulus for agar medium: $y = 0.0218430 - 0.0184750X + 0.1540227X2 - 0.0106765X3 + 0.1295874(X - 0.011)3 + 0.0300795(X - 0.014)3 + 0.0427750(X - 0.017)3$ where y denotes the predicted stiffness of agar medium and X represents the concentration level of the agar medium. The above model is statistically significant with p-value <0.0001 adjusted R-square value of 70%.

Roots elongation in agar with different mechanical strength

We then examined the effect of agar rheology by growing Arabidopsis roots vertically downward in 1.0%−2.0% agar media. When Arabidopsis roots living in an agar medium with increasing concentration, root trait responses to agar stiffness include alterations to the length and straightness. Average lengths of roots from 4-day-old to 7-day-old were varied in different agar concentrations. Root length increased with increasing concentration of agar up to a certain amount, but the excessive addition of agar decreased root length (Figure 4). An equation showed the relationship between 7-day-old root length and agar concentration:

$y = 40.114x3 - 195.12x2 + 304.88x - 136.89$

On the 4th day, the roots in 1.1% agar (Young's modulus = 0.0990 MPa) reached the maximum length (10.02 ± 1.39 mm), and roots in 1.2%, 1.3% agar were almost 10 mm, which were 9.83 ± 0.94 mm and 9.92 ± 1.21 mm, respectively. On the 7th day, the roots in 1.3% agar (Young's modulus = 0.0686 MPa) reached the maximum length (21.43 ± 2.59 mm). There was no clear correlation between root length and Young's modulus.

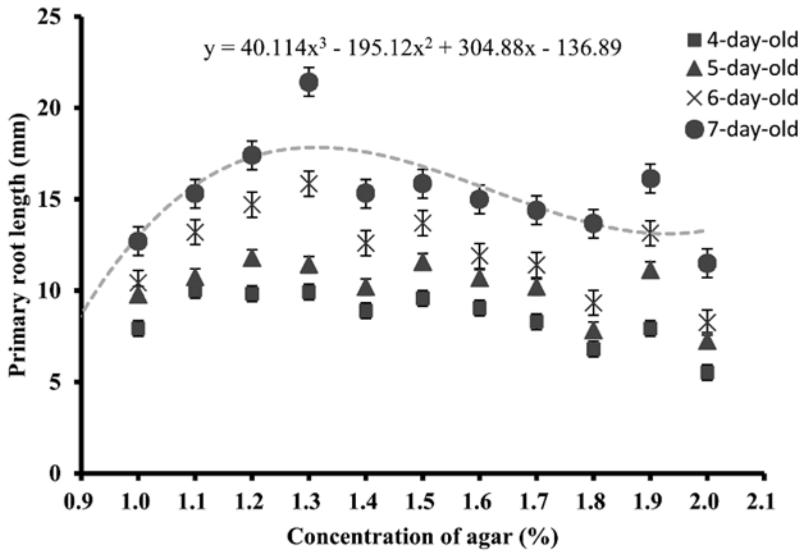

Figure 4: Root length of Arabidopsis from 4 to 7 days old in 1.0%−2.0% agar media, showing the relationship between root length and agar concentration. The model (dashed line) showed the relationship between 7-day-old root length and agar concentration (p-value <0.001, adjusted R-square value of 70%, n = 30). Values represent the mean ± SD.

Root length was decreased with the increase of fracture stress of agar. After 7 days of growth, plants in low strength (1.3% agar, fracture stress = 0.0134 MPa) developed roots averaging 21.43 mm in length while plants in high strength (2.0% agar, fracture stress = 0.0273 MPa) of the same age developed roots averaging 11.50 mm in length. Correlation analysis indicated that there was a significant negative relationship between 7-day-old root length and agar fracture stress, as shown in **Figure 5.**

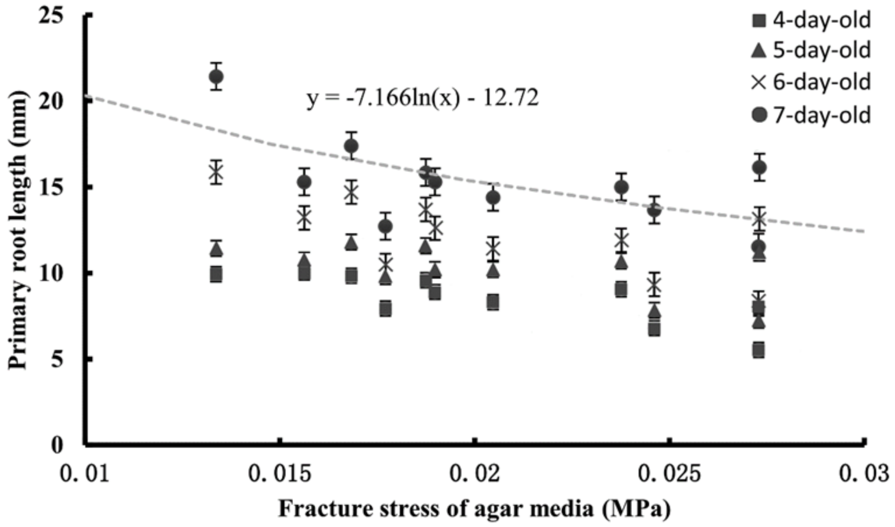

Figure 5: The relationship between root length from 4 to 7 days old and fracture stress of 1.0−2.0% agar. The model that fracture stress inhibited root elongation (dashed line) is statistically significant with a p-value <0.01 adjusted R-square value of 60%. n = 30. Values represent the mean ± SD.

For quantification of root phenotype, we analyzed root straightness. Higher straight-ness values indicate less curved, and lower values indicate more curved. When grown vertically inside the agar medium with low Young's modulus, the primary roots grew with a slight curve. Roots in the agar of 0.0204 and 0.0413 MPa grew almost straight down with straightness > 0.92, and they had a statistically significantly higher straightness value than roots in 0.0435−0.0449 MPa. The straightness decreased as the agar Young's modulus increasing from 0.0204 to 0.0990 MPa. The straightness of the 7-day-old root in different Young's modulus (different agar concentrations) was significantly different **(Figure 6).**

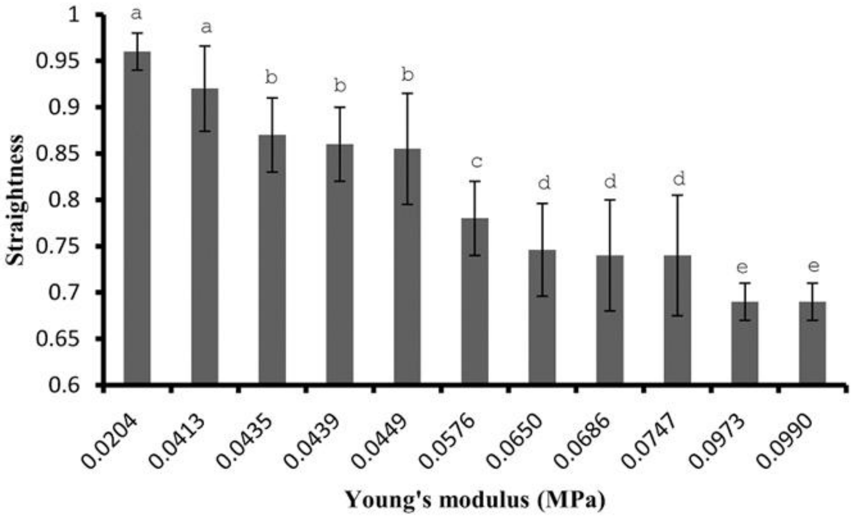

Figure 6: Straightness of 7-day-old Arabidopsis primary roots living in 1.0−2.0% agar media with different Young's modulus. n = 30. Values represent the mean ± SD. Different letters above the bars indicate mean values that are statistically significantly different from one another, according to one−way ANOVA ($P < 0.05$).

Agar rheology characteristics

Dynamic mechanical properties of 0.5%−2.0% agar concentration was detected by the uniaxial compression test. All tested agar media consistently indicate that the addition of agar markedly affects the resistance to fracture and damage tolerance **[19].** Fracture load increased monotonously with increasing agar concentration, indicating that agar can im-prove the mechanical strength of the root culture medium. Our results seem to suggest that the incorporation of agar in 1/2 MS medium was regarded as a good mechanical structure. Kawai et al. **[20]** showed a positive correlation between chain density of polymeric gels and fracture stress. The addition of agar increased the chain density with in-creasing agar concentration, which explains the increase in fracture stress **[21].** The chemical reagents in the medium weaken or strengthen the gel-forming ability of agar. For ex-ample, some salts in 1/2 MS are known to prevent the gelation of agar (Shimizu & Matubayasi, 2014), which may explain the nonlinear increase of agar medium strength with agar concentrations.

Because the breaking load reflects the maximum force loading on the surface of samples when samples are broken, we anticipate that Young's modulus should provide more information for the stiffness or elasticity of agar media. The general tendency of Young's modulus to change with increasing concentration of agar was unexpected, as has been thought in previous papers. Increasing agar concentration from 0.5% to 1.1%, the fracture stress and Young's modulus increased quite well. Therefore, these levels could be used for applying gradual force in plant biomechanical experiments. The results of high agar con-centration (1.2%−2.0%) shown in this study consistently indicate that the addition of agar markedly affect Young's modulus in a non-monotonic manner [19], which suggests that several processes may interplay.

Young's modulus reflects the ratio between stress and strain. Fracture stress of agar media depended on agar concentration. However, agarose gels, as a critical component of agar, have been reported that their fracture strains did not depend on agarose concentration in the concentration range from 1% to 3% [22]. Besides, the relation between fracture strain and agar concentration is more complicated because sucrose in agar medium increased the elastic modulus and the fracture strain of agar gels [19,21]. Sucrose in the agar medium has a network strengthening effect, but when the concentration is above 1.0%, the dramatic increase in viscosity could inhibit network formation. Synthesizes the above analysis, we could answer why the variation of Young's modulus is not monotonic.

Root-gel interactions

The composition of the agar culture medium can change its rheological properties and change the growth behavior of the plant. Studying the mechanical properties of agar and exploring the root response to mechanical stimulation of agar can help establish the root sensitivity to various mechanical properties. Based on our results, we proposed that the strength of 1.1%−1.3% agar was very appropriate for the root formation in the early stage and elongation in the late phase **(Figure 4).** The reasons were that those agar levels would not significantly enhance extrusion force for root elongation **(Figure 3)** when they grew in it and might provide suitable mechanical stress for root development [6,9,23]. The force on each specimen linear increased with the rising of displacement, implying that each agar medium was linearly elastic, homogeneous, and isotropic. Because the primary root was tiny, and the root tip was treated as a rod. Root tip has been regarded as a homogeneous column, and it is linearly elastic, isotropic, and incompressible [24,25]. Arabidopsis roots were cultivated in these agar media with gradient strength, which leads to gradient resistance during root penetration. In root length results, as agar concentration increased, the root growth rate decreased proportionately to the mechanical resistance. Previous studies considered basal medium, plant growth regulators, and agar concentration as factors affecting plant initiation frequency [26]. However, rather than the chemical properties of the basal medium, root elongation was strongly linked to differences in the physical property that result from agar concentration [3,27]. Root straightness was mainly influenced by agar stiffness (Young's modulus), supporting the hypothesis that when resistance in medium increase, growth pressure push root apex to break the gel in front more difficultly, and deflections of root increase [28]. Analysis of root-gel interaction revealed that mechanical force plays a critical role in root elongation, which consisted of a previous study of other f Medicago truncatula plants [24]. From the published literature of plant morphogenesis, the cause of the formation of phenotype could be estimated from agar rheology data [27]. Our results demonstrated that direct contact between root and agar resistance influences root development. Thus, roots, even whole plants are sensitive to the characteristics of the growth medium.

Conclusions

We investigated the mechanical properties of 0.5%−2.0% agar media, based on which we designed a root elongation experiment in Arabidopsis. Nonlinear increase of medium rheology with agar concentrations is a novel finding. The results can make improvements for further investigations on the root development and elongation regulated by agar rheology.

Materials and Methods

Agar medium preparation

The medium consisted of 0.5× MS basal salts (Phyto technology laboratories M519), 15 g sucrose l-1, and 0.5%−2.0% agar (molecular weight 3000−9000, gelling temp. 26−28 °C, Sigma, A4675), and was adjusted to pH 5.8 with 1 mM KOH. Agar consists of about 70% agarose and 30% agaropectin, the agarose, a neutral gelling fraction, consists of a linear polymer of alternating D-galactose 3,6-anhydrogalactose units. The agaropectin, a non-gelling fraction, consists of β-1,3-glycosidically linked D-galactose units, some of which are sulfated at position 6 (Sigma Chemical Co. Plant Culture Catalog). A total of 5−20 g agar was dispersed in 1 L of 1/2 MS liquid medium, and the mixture was heated with stirring at 90 °C until the agarose was dissolved completely. The melted agar media were sterilized by high-pressure steam (1.05 kg/cm, 121.3 °C for 20 minutes). Homogeneous hot agar media with a series of concentrations from 0.5% to 2.0% were poured into the Petri dishes (Diameter = 9 cm, glass) with a thickness of 12 mm. Specimens for the com-pression test were obtained curing for one night. The agar gels were cut to dimensions of 20 mm × 40 mm with a thickness of 12 mm. These rectangle specimens were under 22 ± 2 °C and saturation moisture content, which was identical to the preparation of plant culture medium. All investigations were repeated in triplicate, and at least six specimens were measured from each preparation.

Uniaxial compression test

Uniaxial compression experiment was performed on an Electron Puls E1000 ma-chine (Capacity, 1000 newtons) using Console Bulehill 3 software, and began when the load force touch the sample top area and reached 0.001 N. Loading rate was 1 mm/minute, load force increased at the beginning, reached the maximum, then declined. The test was finished when the load force starts to decline. In this study, various agar amounts have been used to regulate the mechanical properties of the plant growth medium. Agar media were tested from 0.5% to 2.0%. A minimum of 6 specimens of each agar concentration was tested to obtain average values for fracture load, fracture strain, and Young’s modulus. The agar medium is very brittle and could not be measured by a uniaxial tensile test.

Root growth in agar media

Because Arabidopsis roots grew in 0.5−0.9% agar media develop in a spiral pattern [20], we measured roots in 1.0−2.0% agar from 4-day-old to 7-day-old. Wild-type Columbia Arabidopsis (Col-0) seeds were surface-sterilized with 70 % ethanol for 1 minute and a half-strength bleach for 10 min. Sterilized seeds were cold treated at 4 °C for 2 days before being sowed in the growth medium. Pour the melted, sterilized agar media into the Petri dishes (diameter = 9 cm, glass) with a media thickness of 12 mm. When the agar cooled and hardened, the agar was cut along the chord (8 cm) with a scalpel, and the small part was discarded. Place the Petri dishes vertically, and make sure the chord horizontally. Seeds were sown 15-20 per petri dish in a horizontal row, into the medium 1 mm deep. The plates were sealed with porous micropore surgical tape. Additional growth conditions included continuous light (100 μmol m-2s-1, cool white fluorescent, and incandescent bulbs) and 75% relative humidity at 23 °C.

Statistical analysis

Multiple samples with various ratios of agar were used in the compression test. The first peak of the compression curve was analyzed to calculate the fracture stress for agar mechanical strength. Instantaneous fracture stress (σ f) was obtained by

$$\sigma_f = F / A_1 \tag{1}$$

where A1 is the cross-section area at fracture, and F is the loading force at fracture.

Young’s modulus (E) describes compression elasticity or stiffness (the tendency of an object to deform along an axis when compressional forces are applied along that axis). It is defined as the ratio of positive stress and the positive strain of elastic material under stress. We calculated E by

$$E = \frac{\text{STRESS}}{\text{STRAIN}} = \frac{\sigma}{\varepsilon} = \frac{F / A_0}{\Delta L / L_0} = \frac{F L_0}{A_0 \Delta L} \tag{2}$$

where:

E: Young's modulus;

F: the force loaded on a sample under compression;

A0: original cross-sectional area through which the force is applied;

ΔL: the amount by which the length of the object changes;

L0: original length of the object.

Thirty roots were tested for each medium, and experiments were typically repeated more than three times. Images of the growing roots were generated by photographing the individual plates. The root growth was analyzed by image J. Root lengths were deter-mined from 4 to 7 days old seedlings, and straightness (S) was used to quantify buckling as described previously [21], that a length of cord connecting the base and apex of a primary root (Lc) normalized onto the length of a root (L): S= Lc/L. Results are presented as mean ± standard deviation. Statistical analysis was carried out using one-way ANOVA. Linear equations were obtained using the least-squares method.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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Conflicts of Interest: The authors declare no conflict of interest.

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