

Journal of Visualized Experiments

Biomimetic replication of root surface microstructure using alteration of soft lithography

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61437R1
Full Title:	Biomimetic replication of root surface microstructure using alteration of soft lithography
Section/Category:	JoVE Biology
Keywords:	Root; Surface; Microstructure; Synthetic; Biomimetic; Interaction
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the city, state/province, and country where this article will be filmed. Please do not use abbreviations.	Rishon Lezion, Israel

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Dr. Nandita Singh, Senior Science Editor
JoVE

Thursday, March 5, 2020

Dear Nandita:

Following our conversation, I am pleased to submit a method article entitled “Biomimetic replication of root surface microstructure using alteration of soft lithography” for consideration for publication in *JoVE*.

In this manuscript, we develop a method for the replication of plant root surface microstructure, specifically a tomato plant, to a synthetic material. Surface microstructure is known to have a wide effect on the behavior of microorganisms on said surface. This has been previously shown with synthetic leaf surfaces, enriching our understanding of the complex relation between microorganisms and the leaf surface. However, methods for studying the structural effect in root-microorganism interaction, isolating it from all other chemical and molecular parameters of the natural system, are scarce. Hence, the development of a method addressing this issue is of high importance. Here, we develop such a method for the first time, extrapolating the work done on leaves to address the specific challenge of root surface microstructure replication.

The development of this method was recently published in *Plant and Soil* but all the text and figures in this submission have never been published. This method could be of great use for researchers studying root-environment interactions. It is therefore necessary for them to be able to follow the procedure, and I believe visualization of this protocol is the best course of action. I am excited to submit this manuscript to *JoVE* and am hopeful that visualization of this method will encourage many researchers to use it, by that opening a new route in the study of the root's interactions with the surrounding.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

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Thank you for your consideration.

Sincerely,

Maya Kleiman, PhD
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TITLE:

Biomimetic Replication of Root Surface Microstructure using Alteration of Soft Lithography

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KEYWORDS:

Root, Surface, Microstructure, Synthetic, Biomimetic, Interaction

SUMMARY:

Biomimetics has been previously used as a tool to study leaf-microorganism interactions. However, no such tool exists for roots. Here, we develop a protocol to form synthetic surfaces mimicking root surface microstructure for the study of root-environment interactions.

ABSTRACT:

Biomimetics is the use of chemistry and material sciences to mimic biological systems, specifically biological structures, to better humankind. Recently, biomimetic surfaces mimicking the microstructure of leaf surface, were used to study the effects of leaf microstructure on leaf-environment interactions. However, no such tool exists for roots. We developed a tool allowing the synthetic mimicry of the root surface microstructure into an artificial surface. We relied on the soft lithography method, known for leaf surface microstructure replication, using a two-step process. The first step is the more challenging one as it involves the biological tissue. Here, we used a different polymer and curing strategy, relying on the strong, rigid, polyurethane, cured by UV for the root molding. This allowed us to achieve a reliable negative image of the root surface microstructure including the delicate, challenging features such as root hairs. We then used this negative image as a template to achieve the root surface microstructure replication using both the well-established polydimethyl siloxane (PDMS) as well as a cellulose derivative, ethyl cellulose, which represents a closer mimic of the root and which can also be degraded by cellulase enzymes secreted by microorganisms. This newly formed platform can be used to study the microstructural effects of the surface in root-microorganism interactions in a similar manner to what has previously been shown in leaves. Additionally, the system enables us to track the

microorganism's locations, relative to surface features, and in the future its activity, in the form of cellulase secretion.

INTRODUCTION:

Replication of leaf surface microstructure is a known method in the biomimetics research field¹⁻⁴. The earliest replications of the leaf surface microstructure were performed using nail polish and rubber materials applied on the leaf surface for better visualization of microstructure, specifically stomata⁵⁻¹⁰. The method was then improved, and advanced polymers were used to mimic leaf surface microstructure using soft lithography, especially in the context of biomimetics of super hydrophobic surfaces^{2-4,11,12}. In recent years, this method was proven as a useful tool in the study of the interaction between the leaf surface and microorganisms residing on the surface whether they are pathogenic^{13,14} or beneficial, as part of the natural leaf phyllosphere¹⁵. Simplification of the natural system was proven extremely useful in the study of surface-microorganism interactions even when purely synthetic systems were used as surfaces¹⁵⁻¹⁸.

While replication of leaf surface microstructure was shown to be a useful tool for studying the interaction occurring on the surface of the leaf with different microorganisms, no such tool exists for plant roots. Plant roots are harder to study since they reside below the ground and all interactions occur within the soil. Similar to leaves, root surface microstructure is likely to play a role in root-microorganism interactions. However, currently no method exists to isolate the specific role of root surface microstructure in the complex root-microorganism interactions. The most studied root surface microstructural feature is the root hairs¹⁹⁻²¹. Root hairs have an important role in increasing the surface area and by that allowing more efficient intake of nutrients and water²², however their involvement as a structural feature in root-microorganism interactions has never been tested.

The most widely used polymer for soft lithography in leaves is polydimethyl siloxane (PDMS). PDMS properties resemble those of the leaf cuticle^{15,23}. However, in plant roots, the most abundant material is cellulose^{24,25} which has different properties than those of PDMS²⁶⁻²⁸. Using PDMS to build a synthetic platform for studying the surface microstructure effects in root-environment interactions is, hence, less than ideal.

The protocol presented here enables the formation of synthetic root surface microstructure replica from various materials. Like the method for leaf surface microstructure replication this is a two-step process. The first step uses the biological tissue (root) as a source for molding into a polyurethane mold (a negative replica). The polyurethane mold, which represents the negative image of the root surface microstructure, can then be used as a base to generate the positive replication of the root surface microstructure from a variety of materials, including PDMS and cellulose derivatives. This root surface replication can later be used as a platform to understand the surface structure role in root-microorganism interactions.

PROTOCOL:

1. Growing the plants and root preparation

88
89 1.1. Option 1: Prepare adventitious roots from stem.

90
91 1.1.1. Take a rooting tray for growing plants.

92
93 1.1.2. Fill the tray with soil.

94
95 1.1.3. Add one seed of M82 tomato cultivar to each cell in the tray.

96
97 1.1.4. Cover the seeds with a little soil.

98
99 1.1.5. Water the tray from the bottom with a dropper as the water fills the bottom of the tray
100 and the soil absorbs water.

101
102 1.1.6. Add 2 mL of fertilizer per 1 L of water to the bottom of the tray once a week.

103
104 1.1.7. Grow in a growing chamber at 25 °C.

105
106 1.1.7. Use lighting conditions of 9 h light (7:00-16:00) alternated with 15 h of darkness.

107
108 1.1.8. After 3 weeks remove the plant from the soil.

109
110 1.1.9. Cut the root system from the plant at the point of interaction with the stem.

111
112 1.1.10. Put the rootless plant in a beaker filled with water.

113
114 1.1.11. After a few days, cut the adventitious roots that emerge from the stem and use them for
115 replication.

116
117 1.2. Option 2: Prepare seed germinating roots.

118
119 1.2.1. Wet a Petri dish sized filter paper with water.

120
121 1.2.2. Put several M82 seeds (no more than 10) on the paper, inside a Petri dish.

122
123 1.2.3. Incubate the plate at 25 °C.

124
125 1.2.4. Hydrate the paper every day.

126
127 1.2.5. After germinated roots are long enough (approximately 5 days), remove the seeds and use
128 the roots for replication.

129
130 2. Preparation of the root negative replica from polyurethane
131

2.1. To generate negative replica solution, add 9.49 g of diurethane dimethacrylate to a 20 mL vial.

2.1.1. Add 1.45 mL of ethyl methacrylate to the vial.

2.1.2. Stir at room temperature (RT) until the solution looks clear and becomes homogeneous.

NOTE: Approximately 2 h is sufficient to reach a homogeneous solution.

2.1.3. Add 3 mL of the plasticizer, diethyl phthalate, and stir for 1 h at RT.

NOTE: Diethyl phthalate is miscible in acrylate monomer.

2.1.4. Add 300 μ L of the photo initiator, 2-hydroxy-2-methylpropiophenone, and stir overnight at RT. Continue stirring until all bubbles are removed.

NOTE: The protocol can be paused here. The solution can be kept at RT.

2.2. To generate the negative replica of the root, take a clean glass slide and pour 1 mL of the negative replica solution on it.

2.2.1. Place 2–3 roots over the solution. Do not allow the roots to be fully covered by the solution.

2.2.2. Keep the slide under 8 W ultra violet (UV) lamp for 8–10 min. Do not keep the solution under UV light for too long.

NOTE: It is important not to keep the solution under the UV light for too long as it makes the polyurethane too hard, making it impossible to remove the root.

2.2.3. Switch off the UV lamp, remove the replica from the glass slide and put it in a Petri dish filled with ethanol, to remove unreacted monomer.

2.2.4. To obtain the negative replica, remove the root from the replica very slowly using forceps.

3. Prepare the root positive replica from PDMS.

3.1. To generate the mixture for the positive replica, place 10 g of dimethyl siloxane in a paper cup.

3.1.1. Add 1 g of curing agent and mix thoroughly.

3.1.2. Keep the mixture in a desiccator under vacuum for 2 h to remove air bubbles.

3.2. To generate the positive replica, place the polyurethane negative replica in a Petri dish.

3.2.1. Pour the PDMS mixture on top of the negative replica.

3.2.2. Apply vacuum for 2 h to assure coverage of the microstructure.

3.2.3. Keep the Petri dish overnight at RT.

3.2.4. Separate the cured positive replica from the negative replica by hand.

4. Prepare the root positive replica from ethyl cellulose.

4.1. To generate the ethyl cellulose solution, put 1.4 g of ethyl cellulose in a 20 mL glass vial.

4.1.1. Add 20 mL of ethanol and stir at RT for 2 h.

4.1.2. Add 6 mL of diethyl phthalate as a plasticizer (30 % of the solution) and stir overnight.

4.2. To generate the positive replica, place the polyurethane negative replica in a Petri dish.

4.2.1. Pour the ethyl cellulose solution on top of the negative replica.

4.2.2. Keep the Petri dish overnight at RT under the hood.

4.2.3. Remove the positive replica from the negative replica very slowly by forceps.

REPRESENTATIVE RESULTS:

To form the root surface microstructure replication, a root must be chosen for molding. We grow tomato plants in soil, making the use of the natural root from the root system extremely challenging. Removal of soil from the root system can be difficult and additionally, the root system roots are fragile and can break upon molding attempts. We therefore suggest to first use more rigid roots, to establish the protocol in the lab. The formation of such roots is described in **Figure 1A**. The plant root system is removed after the plant was grown for 3 weeks and the rootless plant is placed in water for about a week until adventitious roots emerge from the stem. Those roots can be used for replication during the protocol establishment. Once the protocol has been well established, a more realistic root surface structure is desired. Here we suggest avoiding roots grown in soil as the full removal of soil is extremely challenging. Instead we suggest the use of germinating roots, supplying valuable information on the root surface microstructure of a genetically specific plant. The growth of such roots is described in **Figure 1B**. The seeds are placed on a wet filter paper and incubated at 25 °C. After approximately 5 days, during which the filter paper is kept moist, the germinated roots are long enough for replication. Those roots are more fragile than the previously suggested roots and require more delicate care.

The production of the root surface microstructure replica is a two-step process. In the first step the natural root is being molded into a polyurethane based mold (the negative replica). The advantage of this step is that all materials for the polyurethane mold are being prepared and the

root is placed on top of the prepared solution at the very end for a 10 min exposure to UV. As a result, the biological tissue is not exposed to harsh conditions for too long and can be gently handled at the end of the process. If all protocol steps are followed, a good negative replica is generated. This replica will show the cell structure of the root surface as well as holes representing the location of the root hairs (**Figure 2A**). If some critical steps in the protocol are not being followed, the procedure will fail. One such step is the placement of the root on the polyurethane solution prior to curing. The root must be placed very gently to avoid the submergence of it in the polyurethane solution. Such submergence, of any part of the root, will cause the entrapment of the root in the hard polymer with no ability to remove it. If such an event occurs, the root will remain within the negative replica after it is cured (**Figure 2B**). Another crucial step is regarding the curing time by UV light. The recommended curing time is 8–10 min. Going past 10 min will result in an extremely hard polyurethane mold, making it impossible to remove the root without breaking it within the polyurethane mold. The breakage of the root can sometimes be visible to the naked eye, e.g., when a large piece is broken (**Figure 2C**, marked with purple arrows). However, sometimes small root pieces are left in the material which are difficult to spot by the naked eye and a microscope has to be used (**Figure 2C**, marked with purple arrows). We recommend carefully examining the polyurethane negative replica with a microscope prior to the continuation of the protocol to make sure no residual root is present.

Once the polyurethane negative replica is prepared; many materials can be used for the preparation of the positive replica. The preparation of the positive replica, using the polyurethane negative replica as a mold, is straight forward and depends completely on the quality of the polyurethane negative replica. To generate the positive replica we have used both PDMS—as it is well known in the field of soft lithography (**Figure 3A**)—and ethyl cellulose as a material that better mimics the properties of the root surface which is mostly composed of cellulose (**Figure 3B**). The SEM image of the PDMS replica shows the root hairs very clearly. The hairs are in the elongation zone, where they begin to emerge. Hence, the length of root hairs varies along the root surface as they become longer, much like in the natural root (**Figure 3A**). Ethyl cellulose generates harder and less flexible film than PDMS. Hence, the removal of it from the negative mold requires more care. However, some hairs and the surface microstructure are visible under the light microscope (**Figure 3B**). We used those two materials to generate the positive replica, however, any material that can form a film will be a good candidate for the positive replica, using the polyurethane negative replica.

FIGURE AND TABLE LEGENDS:

Figure 1: Tomato plant roots for replication. (A) A tomato (M82) plant is grown at 25 °C with 9 h of light and 15 h darkness. After 3 weeks, the plant is removed from the soil and the root system is cut off. The rootless plant is put in water until adventitious roots emerge from the stem after about a week. These roots do not show the exact structure as the roots from the root system, but they represent a good model. Those roots are less fragile than the root system roots and hence are preferred to work with when establishing the technique in the lab. (B) Tomato (M82) seeds are put on a wet filter paper in a Petri dish and incubated at 25 °C. The paper is hydrated every day and the seeds are germinating. The roots are growing and after approximately 5 days

are long enough to be used for replication. These roots are gentler and should be used once the method is well established.

Figure 2: Microscopy images of polyurethane negative replica. (A) SEM image of polyurethane negative replica made according to a protocol following all the steps. Cell structure is clearly visible. Yellow arrows point at holes formed by the hairs in the root. (B) Light microscopy images of polyurethane negative replica with a root inside of it as it was fully covered with the solution and the removal of it was impossible. The polyurethane negative was cured with the root inside. The root is visible by eye and using light microscopy. It is impossible to remove this root from the cured replica. (C) Light microscopy images of polyurethane negative replica that was kept under UV light for too long. As a result, root could not be fully removed from the polymer with either large particles visible by eye (top image, marked with purple arrows) or small fractions visible only by microscope (lower image, marked with purple arrows).

Figure 3: Microscope images of positive replica. (A) SEM micrograph of a positive replica made from PDMS. Enlargement shows root hairs. (B) Light microscopy images of a positive replica made from ethyl cellulose. Hairs are shown in the images on the right while surface texture is visible in the image on the left.

DISCUSSION:

We present a novel method for the replication of root surface microstructure. This method relies on existing methods of leaf surface microstructure replication⁴. In order to develop this method, we had to tweak the existing method for leaves. We realized that the problematic step in copying the leaf replication method into roots involves the first step of the root molding. This is the most sensitive part of the method as it involves the biological tissue. As a result, we wanted to choose a polymer that would demand relatively gentle conditions for curing and hence causing minimal damage to the biological tissue. We chose polyurethane because it can be polymerized quickly (within 10 min) under UV light²⁹. Additionally, it is very hard once polymerized³⁰ and we hoped that this property would allow for the relatively easy removal of the root from the polyurethane mold.

The presented method is a two-step approach in which the negative image (negative replica) is formed in the first step and the replication is formed in the second step, based on the negative replica. This extends the range of materials we can work with. Leaf surface microstructure replication was mainly performed on PDMS or epoxy materials^{11,31}. Some work was done with other materials, specifically materials supporting microorganism growth^{13,32}. This is because in recent years this method has been used to study microorganism-surface interactions in the context of leaf surface structure. However, no cellulose-like materials have been used in this method in the context of leaves. We suggest the use of a polyurethane negative replica as a mold and a variety of materials for the positive replica. In other words, making the positive replica, from a variety of materials, is relatively easy once a good negative replica is made. We currently use cellulose derivatives, but are exploring the possibilities of using more relevant materials to root surface such as pectin and lignin^{33,34} in combination with cellulose derivatives.

The method also expands upon the existing method of leaf surface microstructure replication since the leaf is a 2D surface while the root surface is curved and hence is a 3D surface. Our method does not enable the replication of the whole surface since embedding the whole root in the polyurethane solution does not allow for its release. Therefore, one side of the root has to be chosen when replicating the root surface microstructure. The generated synthetic surface is curved and represents roughly half the surface, but not all of it. Our assumption is that the structural features of the root surface are mostly symmetrical about the axis along the root length. However, in studies where such symmetry is not assumed, one should be careful to choose the appropriate side root to replicate.

We present two options for roots to be used as molds. The first is the option of adventitious roots grown from the stem and the second is the option of germinated roots on paper. The first option is mostly meant to assist researchers in practicing the method as these roots are more robust and easier to work with. The second option represents the genetic differences that can be found between roots of different cultivars, regardless of the environmental conditions. These surfaces can be used as important research tools, however, one should be aware that the environment can have a strong influence on the root surface structure, specifically the soil in which the roots are grown^{35,36}. Due to the mechanical stress inflicted by the soil, some morphological changes are bound to happen, in addition to wounds accruing on the surface as the root penetrates the soil³⁷. Removal of roots from soil, as well as cleaning them, without damaging their structure is a very difficult task. Hence, we are not optimistic as to the ability to use this method to reliably mimic the root surface microstructure of roots grown in soil. However, for research that focuses on genetic differences or environmental differences where the change in microstructure is noticeably clear, this method can be used as a tool to study the influence of root surface microstructure.

Our method produces an inert surface mimicking of only the microstructural properties of the root surface. While this method is designed to separate the structural effects in root-environment interactions from all other effects, we cannot ignore the chemical compounds in those interactions. Some microorganisms may not survive or function on the surface without the addition of compounds, specifically nutrients. The next step in the development of this platform will be the controlled addition of chemical compounds to study their effects on the different interactions when combined with structure.

This method was developed as a first step in the development of a synthetic platform to study root-microorganism interactions. Here we mimic the microstructure of the root surface and this initial platform can be used to study the influence of surface microstructure on microorganism behavior. However, this platform is limited since it lacks many other elements from the natural system. This platform should be further developed with the use of the right materials to generate the surface and with the addition of other, critical, chemicals into the system. In a more advanced platform, we can also imagine spatial distribution of the chemicals. However, since currently no other method exists to isolate structural effects in root-microorganism interactions, we hope

researchers could use this initial platform to ask structure-specific questions in those interactions.

ACKNOWLEDGMENTS:

Research was supported by seed funds from The Agricultural Research Organization to MK.

DISCLOSURES:

The authors have nothing to disclose.

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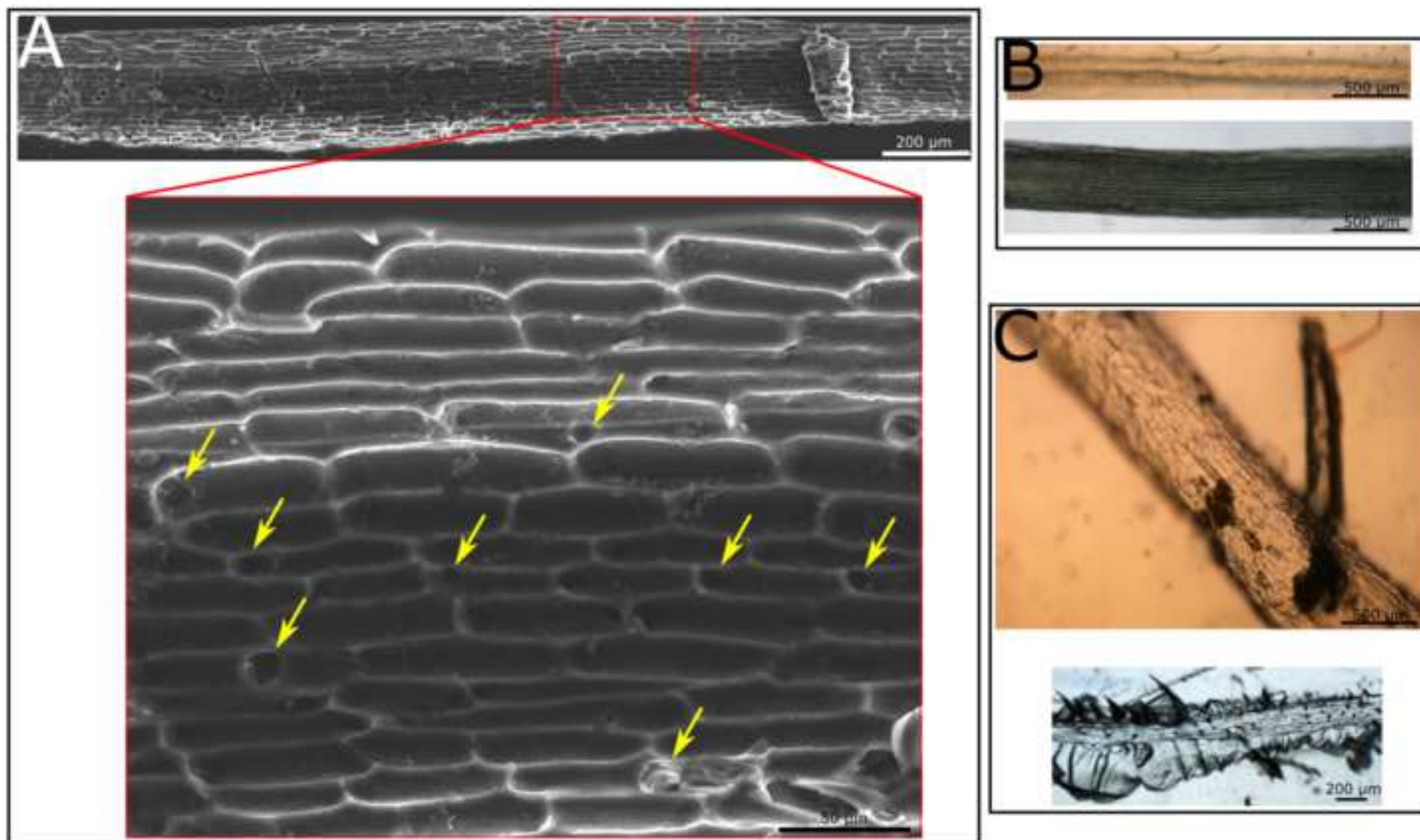
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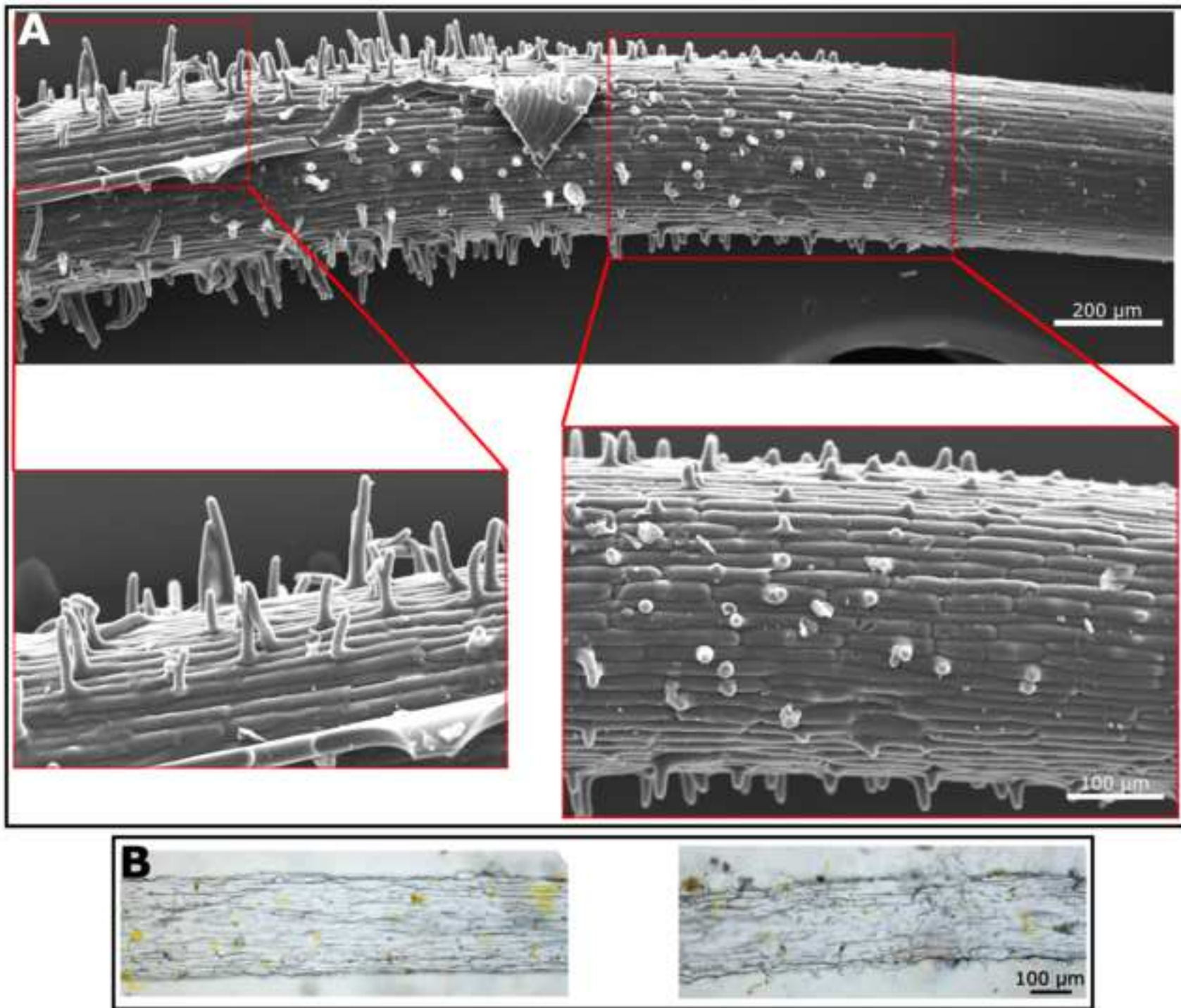
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-hydroxy-2-methylpropiophenone	Sigma	405655	
Diethyl phthalate	Across	114520010	
Diurethane dimetharylate	Sigma	436909	
Ethyl cellulose	Across	232705000	
Ethyl methacrylate	Sigma	234893	
Shaphir Solution	GAT fertilizer	6-2-4	
Sylgard 184 kit	Polymer-G	510018400500	

Response to reviewers regarding submission titled: Biomimetic replication of root surface microstructure using alteration of soft lithography

We thank the editor and reviewers for their comments. In the document below we address all comments.

Editorial comments:

1. The manuscript has been checked for any spelling or grammar issues.
2. All formatting was performed.
3. Long abstract is 247 words and clearly states the goal of the protocol.
4. Protocol numbering was adjusted.
5. All text in the protocol written in the imperative tense.
6. The protocol contains only action items that direct the reader to do something.
7. Individual steps of the protocol only contain 2-3 actions sentences per step.
8. How questions are answered.
9. Only one note follows one step. All notes are used for text that does not provide details about how to perform a particular step.
10. The protocol is less than 2.75 pages. Other than the trivial steps of plant growing, all the protocol is highlighted.
11. No figure was previously published and hence no copyrights need to be obtained.
12. All microscope images have scale bars.
13. Results describe experiment, what we wanted to achieve and inline with the title.
14. Discussion is 6 paragraphs long and addresses the required topics.
15. All journal names in the references are not abbreviated.
16. The table of essential supplies, reagents and equipment is a .xlsx. file that includes name, company and catalog number of all relevant materials in separate columns and in alphabetical order.

Reviewer 1:

We thank the reviewer for mentioning that that the protocol is of interest to many disciplines.

Response to major concerns:

We thank the reviewer for the remark regarding the difference between the known 2D method for leaf replication and our 3D method for root replication. We also agree that since the root is not completely embedded in the polymer and only one side is replicated, there are limitations to this method that need to be addressed. We added a paragraph to the discussion section addressing this point (lines 302-310).

We agree that there are no pictures of the molding process. This is because, since the materials are all transparent, it is very difficult to see anything by simply taking a picture. The relevant pictures are only microscopy images, which we present in the paper. We rely on the video that will make it clearer. In a still picture that shows no movement with transparent materials it is almost impossible to capture the process.

Response to minor concerns:

Figure 3C was changes to Figure 2C on lines 233 and 235 (now lines 231 and 233).

We copy-edited our paper to remove any language mistakes.

Reviewer 2:

We thank the reviewer for this positive review.

Response to major concerns:

We added some discussion regarding the differences between the roots used in this paper and the ones grown in real soil. This was added as an additional paragraph in the discussion in lines 311-325.

We clarified the text explaining Figure 2B both in the representative results text (lines 226-227) and in the figure legends. To clarify Figure 2C, we added purple arrows to the figure, pointing to the residual root left in the replica and mentioned them in the text of both the representative results and the figure legends.

ON was changes to overnight throughout the text.

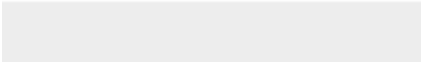

The sentence: “In other words, making the positive replica, from a variety of materials, is relatively easy once a good negative replica is made.” Was added to the discussion in lines 298-299 to amplify this point.


We placed the legends of all sub-figures in separate paragraphs.

We added some reference to the images in Figure 3, including root hairs and the differences between PDMS and ethyl cellulose (lines 242-247).




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