# Lateral mechanical impedance rather than frontal, promotes cortical expansion of roots

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# Abstract

It has long been considered that mechanical impedance on root will restrict root elongation and consequently promote radial growth. However, we did observe radial expansion but not elongation restriction in maize seedlings after short growth in sands. Mechanical impedance of soil can be classified into frontal- and lateral-type based on the interaction site of root. Therefore, we suspected that radial expansion might be mainly stimulated by lateral rather than frontal impedance. To verify our speculation, frontal and lateral impedance was provided separately. Small plastic caps were used to provide unique frontal impedance on root tip and cylindrical plastic containers were used to provide lateral impedance. Plastic caps could reduce root length remarkably. However, the radial expansion of plastic-cap-fitted roots was significantly inferior to that of the sand-cultured roots. Microstructural analysis revealed that sand-condition thickened root largely dependents on cortical expansion,

29   whereas plastic cap did it mainly by thickening stele. In cylindrical plastic containers,

30   mechanical impedance came only from the lateral direction and promoted the

31   expansion of cortex just as sand-condition. Thus, we proposed that the expansion of

32   the cortex and the consequent radial growth is mainly due to lateral impedance when

33   growing in sands.

## Introduction

35       The growth of roots in soil can be limited by the physical, chemical, and

36   biological properties of soil, among which physical properties have been reported to

37   be most strongly linked to root elongation [1-3]. In terms of the physical limitations

38   on root growth, mechanical impedance (caused by soil that is too compact to enable

39   rapid root penetrance) mainly affects root systems by hindering root elongation and

40   concurrently promoting root thickening [1,3,4]. Soil is a dense medium, and it is

41   necessary for root tips to generate sufficiently mechanical force to penetrate through

42   the soil in the absence of continuous pores of a sufficiently large diameter [1,5].

43   Intuitively, thin roots, given their cross section, may penetrate soil easier as they

44   encounter less resistance than do thicker roots. Moreover, thin roots will reorient their

45   growth easily to circumnavigate the obstacle [1,5]. Therefore, it sounds contradictory

46   that roots grow thicker in response to mechanical impedance [1,4,6]. Indeed, previous

47   experimental evidences related to the effect of root diameter on penetration resistance

48   were often contradictory [1,7]. But recently, more studies on different plant species

49   have indicated that thicker roots rather than thinner roots can more effectively

50   penetrate compact soil layers [4,8].

51       Actually, mechanical impedance of soil can be classified into frontal- and

52   lateral-type based on the position of root, and frictional (lateral) impedance between

53   roots and the soil may account for up to 80% of total mechanical impedance [4,9]. All

54   previous studies on mechanical impedance have obtained data from plants in which

55   the entire root system has been embedded in various substrates, such as glass beads,

56   soil, sands, and phytagel [1,3-5,10]. In these studies, it was not possible to distinguish

57   the effects of frontal and lateral impedance. So, It is not entirely clear as to whether

58  frontal mechanical impedance acts as a direct cue to restrict root elongation and

59  promote radial expansion. In this study, the objective was to determine which of the

60  frontal- or lateral- impedance was the main stimuli on radial expansion of root.

# Materials and methods

## Plant materials and growth conditions

63  To produce the seedlings used in this study, we germinated maize seeds in

64  rolled-up germinating test paper, so as to generate seedlings with relatively straight

65  roots of approximately 3 to 5 cm in length. These seedlings were then subjected to

66  different growth conditions (water-, sand-, semi-sand and cap-fitted-conditions).

67  Silica sand with a particle size about 2 mm in diameter was used for sand and

68  semi-sand conditions. The maize seedlings were grown in a greenhouse at

69  approximately 26°C under a 14-h light/10-h dark photoperiod. Schematic diagrams of

70  plastic cap and cylindrical plastic container were showed in Figure 1a and 1b,

71  respectively. Plastic cap was used for cap-fitted condition. It was prepared in the

72  following manner: a rubber tube with a 3 mm inner diameter was cut into 1 cm

73  lengths and one end of the 1 cm rubber tube was blocked. Then, a tiny elastic thread

74  was fixed to two flanks of the plastic cap. When used, plastic cap was fitted on root

75  tip and elastic thread was attached to the raft where seedlings were anchored (Fig 1D).

76  The cylindrical plastic container was used for semi-sand condition. It was prepared in

77  the following manner: first, plastic pipe with a 20 mm inner diameter was cut into 5

78  cm lengths; second, one end of the 5 cm plastic pipe was blocked by a plastic sheet

79  and scotch tape; at last, making a hole in the center of the plastic sheet about 3 mm.

80  When used, root of seedling was buried in the cylindrical plastic container with sands

81  and root tip was exposed to the outside through a hole (Fig 1E). Four kinds of growth

82  conditions were showed in Fig 1.

## Preparation of paraffin sections

84  Segments of root from the root tip and the region (1 cm in length) near the

85   root–hypocotyl junction were collected from three biological replications (5 seedlings

86   were used in each replication) and stored in Carnoy's fixative (ethanol: glacial acetic

87   acid = 3:1) at room temperature for 12 h. The samples were then subjected to an

88   ethanol gradient, consisting of 75% ethanol for 4 h, 85% ethanol for 4 h, 100%

89   ethanol overnight, and 100% ethanol + basic fuchsin for 4 h. Thereafter, the samples

90   were transferred to xylene (two 1 h treatments). The subsequently obtained

91   transparent samples were embedded in melted paraffin for 12 h, after which the

92   paraffin was replaced with new paraffin and saturated for more than 24 h. The

93   samples were then embedded in paraffin using plastic molds. Cross-sections of the

94   embedded roots (with a thickness of approx. 15 µm) were prepared using an RM2255

95   microtome (Leica, Germany). For each replication, 10 slides (2 slides per sample)

96   were prepared and then stained with 1% toluidine blue for 1 min, followed by three

97   washes in double-distilled water. After inspection for breakage and uniformity, two

98   sections of each slide were selected for image analysis.

## Image acquisition and analysis

100   For the investigation of cell length, fresh primary root tips (approx. 0.8 cm in

101   length) were longitudinally sliced by hand and stained with 50 µg/mL propidium

102   iodide for 10 min. Then, the samples were washed twice and observed using a Zeiss

103   LSM 800 confocal laser scanning microscope (Germany). The view field was located

104   up to 400 µm from the root tip border, and images were acquired using an objective

105   lens with a 10x magnification. To obtain the relative full view of the roots after

106   propidium iodide staining, 20 lateral roots of each sample were selected and their

107   images were acquired using the Tiles model of Zeiss LSM 800. Micrographs of

108   sections were acquired using an Olympus microscope (IX73, Japan) fitted with a

109   DP80 digital camera under white light using a 10x objective lens. Images of root hairs

110   were obtained using an Olympus anatomical lens (SZX2-ILLT, Japan) fitted with a

111   DP71 digital camera under white light using a zoom magnification of 6.4x with its 1x

112   objective. Three biological replications were established, 5 seedlings of each

113   replication were used for cell length analysis and 10 seedlings of each replication

114　were used for the analyses of root length and diameter. Images were analyzed for

115　anatomical traits using Image J software. Cross-sectional diameter and stele diameter

116　were measured directly, whereas cortex thickness was determined by subtracting the

117　stele diameter from the cross- sectional diameter. The difference between samples

118　was based on the Student's t-test.

## RNA extraction and PCR

120　　　　To investigate the expression of *Cyclin* genes, which are the marker genes

121　reflecting cell division activity [11, 12], total RNA was isolated from root tips using

122　the TRIzol reagent (Invitrogen) and treated with RNase-free DNase I (Takara). An

123　iScript™ cDNA Synthesis Kit (Bio-Rad) was used to synthesize cDNA, using 3 µg of

124　total RNA from each sample. Subsequently, quantitative real-time reverse

125　transcription PCR (qRT-PCR) was performed using SsoFast™ EvaGreen® Supermix

126　(Bio-Rad) in 96-well optical reaction plates in a Bio-Rad CFX96 Touch™ thermal

127　cycler. Data were processed as described in our previous publication [13].

128　Quantification was performed using the $2-\Delta\Delta Ct$ method [14], where $\Delta\Delta Ct$ is the

129　difference in threshold cycles between specific genes and the reference housekeeping

130　gene eF1a. The primers used for amplification are listed in Table 1.

## Results

## Frontal impedance reduced root length by restricting cell division and elongation

134　　　　At the beginning, maize seedlings were exposed to sand-condition for 3 and 6

135　days to investigate the effect of sands provided mechanical impedance. Water

136　cultured seedlings were used as controls. When compared to controls, 3 days'

137　sand-cultured seedlings did not display obvious changes in root length, but we did

138　observe a substantial expansion of root diameter, particularly in the region within 3

139　cm of the root apex, which was newly developed after transplant (Fig 2A, 2B).

140    However, by prolonging growth time to 6 days, we found a significant reduction of

141    root length under sand-condition (Fig 2A). From this result, it was believable that

142    elongation restriction and radial expansion were not always accompanying.

143    Thereafter, 3 days of treatment was chose. Next, cap-fitted-condition was added and

144    seedlings were treated for 3 days. Small plastic caps were fitted onto root tips to

145    provide unique frontal impedance. After 3 days of growth, root length, cell length,

146    expression of *Cyclin* genes (marker genes reflecting cell division activity) and root

147    diameter, were investigated [11,12]. We found that in plants with plastic caps, root

148    length was reduced by one-third when compared with that of controls. There was,

149    however, no statistically significant difference in root length between the

150    sand-condition seedlings and controls (Fig 2C). At the microscopic and molecular

151    level, we observed that the presence of plastic cap promoted a marked reduction in

152    cell length and down-regulated expression of *cyclin B* (Fig 2D, 2E), thereby implying

153    low cell division activity under frontal impedance. Accordingly, we could deduce that

154    reductions in cell division and cell length contributed to determining a shorter root

155    length under frontal impedance. In addition, we observed that a larger number of root

156    hairs had emerged on the root of sand-condition seedlings than in the cap-fitted

157    seedlings and controls (Fig 2F).

## Frontal impedance increased stele diameter but not cortical expansion

160       As to root diameter, significant differences were observed between three kinds

161    of seedlings (Fig 3, 2C). Notably, the length of plastic-cap-fitted roots was reduced by

162    one-third compared with those grown in sand, although the root diameter was thinner

163    in the cap-fited roots (Fig 3A, 3B). This finding was unexpected and implies that

164    simple frontal impedance has a slight facilitating effect on radial expansion.

165    Furthermore, root tips were sliced using a microtome for microexamination. The roots

166    of sand-condition seedlings displayed remarkable cortical cell expansion compared

167    with the roots of controls and plastic-cap-fitted roots, and there was no difference

168 between controls and plastic-cap-fitted roots (Fig 3A, 3C). In terms of stele diameter,

169 plastic-cap-fitted roots were thicker than both water- and sand-condition roots,

170 whereas there was no difference between water- and sand-condition roots (Fig 3A,

171 3D). These observations indicate that simple frontal impedance did not induce cortical

172 expansion, but it did increase the stele diameter of roots.

## Lateral impedance promoted cortical cell expansion

173

174 Given that frontal impedance conferred by the presence of a plastic cap did not

175 induce notable cortical expansion, the effect of lateral impedance on cortex was

176 examined. Cylindrical plastic containers were used to provide semi-sand-cultured

177 conditions (shown in Fig 4A), in which mechanical impedance was imposed only in a

178 lateral direction. After 3 days of treatment, root diameter and microstructure were

179 analyzed using the mature region (2 cm below the root–hypocotyl junction). The

180 results showed that both sand and semi-sand culturing promoted cortical expansion

181 when compared with the roots of water-condition (Fig 4B, 4D). However, the

182 increment of cortical expansion at this region was smaller than that in the apical part

183 of roots grown in sand-condition (Fig 3B, 4C). This difference could be attributable to

184 that root tip is a newly developed tissue sensing de novo stimuli of lateral impedance

185 after transplant.

## Discussion

186

187 Considerable evidences have amassed to indicate that mechanical impedance

188 can reduce the elongation and promote the radial expansion of roots [1,4,5,10].

189 Nevertheless, all previous studies on mechanical impedance have obtained data from

190 plants in which the entire root system has been embedded by various substrates, such

191 as glass beads, soil, sand, and phytagel [1,3-5,10]. In these studies, it was not possible

192 to distinguish the effects of frontal and lateral impedance, and consequently, root

193 shortening and thickening always appeared concurrently. In the present study, plastic

194 caps were used to provide simple frontal impedance on the root tips. Subsequent

microstructural observations and expression analysis of cell division marker genes indicated that reduced cell division and cell length jointly determined the short root lengths observed under frontal impedance. However, although the presence of plastic caps resulted in seedlings with shorter roots than those of seedlings cultured in sand, we found that the diameter of plastic-cap-fitted roots was smaller than that of sand-condition roots, implying that frontal impedance has a slight facilitating effect on radial expansion. Mechanical impedance has been demonstrated to increase root diameter mainly via the expansion of cortical cells [4,15]. Based on our results, however, simple frontal impedance did not induce the expansion of the cortex, whereas it did increase the diameter of the stele. Thus, we speculate that the cues promoting cortical expansion may arise from lateral impedance, such as frictional impedance. Actually, the frictional resistance between roots and the soil has been reported to account for up to 80% of the total mechanical impedance [4,9]. Vertical pressure is, however, considered an unlikely candidate, because it has previously been reported to constrict radial expansion and compress cortical cells [3,16].

The expansion of cortical cells is controlled by the orientation of cortical microtubules or actin microfilaments and the consequent deposition of cellulose microfibrils [16-18]. This process is also associated with the secretion of mucilage, for instance, the treatment of root cap cells with actin microfilament inhibitor was found to reduce mucilage production, while disorganization of cortical microtubules has been observed to impair the release of mucilage from seed coat secretory cells [17,19]. Thus, cortical cell expansion appears to be associated with the process of mucilage production and consequently overcomes frictional resistance between roots and sand particles to promote root penetration in sands.

## Conclusions

We propose that cortical expansion and the consequent thickening of roots are primarily induced by lateral impedance (friction) rather than frontal impedance when roots grow in sands.

# Acknowledgments

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291    Table 1. Primers used for quantitative real-time reverse transcription PCR

292    (qRT-PCR).

| Genes | Forward primer | Reverse primer |
|-------|----------------|----------------|
| *eF1a* | TGGGCCTACTGGTCTTACTACTGA | ACATACCCACGCTTCAGATCCT |
| *CYC1* | ACGTTGAGGACATCTACACATT | TATTGTGAGGTAGAGCGTTTCC |
| *CYCB1* | TAGTACTCAGCTCCGATTCTGA | ATGTCATCGACCACTTGTCTAG |

293

294    Fig 1. Schematic diagram of treatment conditions. (A) Root tip in plastic cap. (B) root

295    in cylindrical plastic container. It is water-, cap-fitted-, semi-sand and sand-condition

296    from (C) to (F) successively. Black points in (B) and (F) represent silica sands.

297    Fig 2. Frontal impedance reduced root length by restricting cell division and

298    elongation. Root length (A) and diameter (B) after 3 or 6 days of growth in water-

299    (W) and sand- (S) conditions. (C) Root length after 3 days of growth under W, S, and

300    plastic-cap-fitted (PC) conditions. (D) Confocal micrographs of primary root tips after

301    propidium iodide (PI) staining (left: W, right: PC) and cell length after 3 days of

302    growth under W and PC conditions. The yellow scale bars represent 100 μm. (E)

303    Expression level of two *Cyclin* genes (cell division    marker genes) and *eF1a* as an

304    internal control. (F) Root hairs on primary root viewed under an anatomical lens

305    (from left to right: W, S, and PC). **$P < 0.01$; *$P < 0.05$; ns: non-significant.

306    Fig 3. Root diameter (A), cortical thickness (B) and stele diameter (C) of root tips.

307    Micrographs of a cross-section under water-, sand- and plastic-cap-fitted conditions

308    from (D) to (F) successively. The red scale bars represent 100 μm. **$P < 0.01$; *$P <$

309    $0.05$. W, S and PC represent water-, sand-, and plastic-cap-fitted conditions,

310    respectively.

311    Fig 4. Root diameter (A), cortical thickness (B) and stele diameter (C) of mature

312    region. Micrographs of cross-sections under water-, sand- and semi-sand- conditions

313    from (D) to (F) successively. The red scale bars represent 100 μm. **$P < 0.01$. W, S

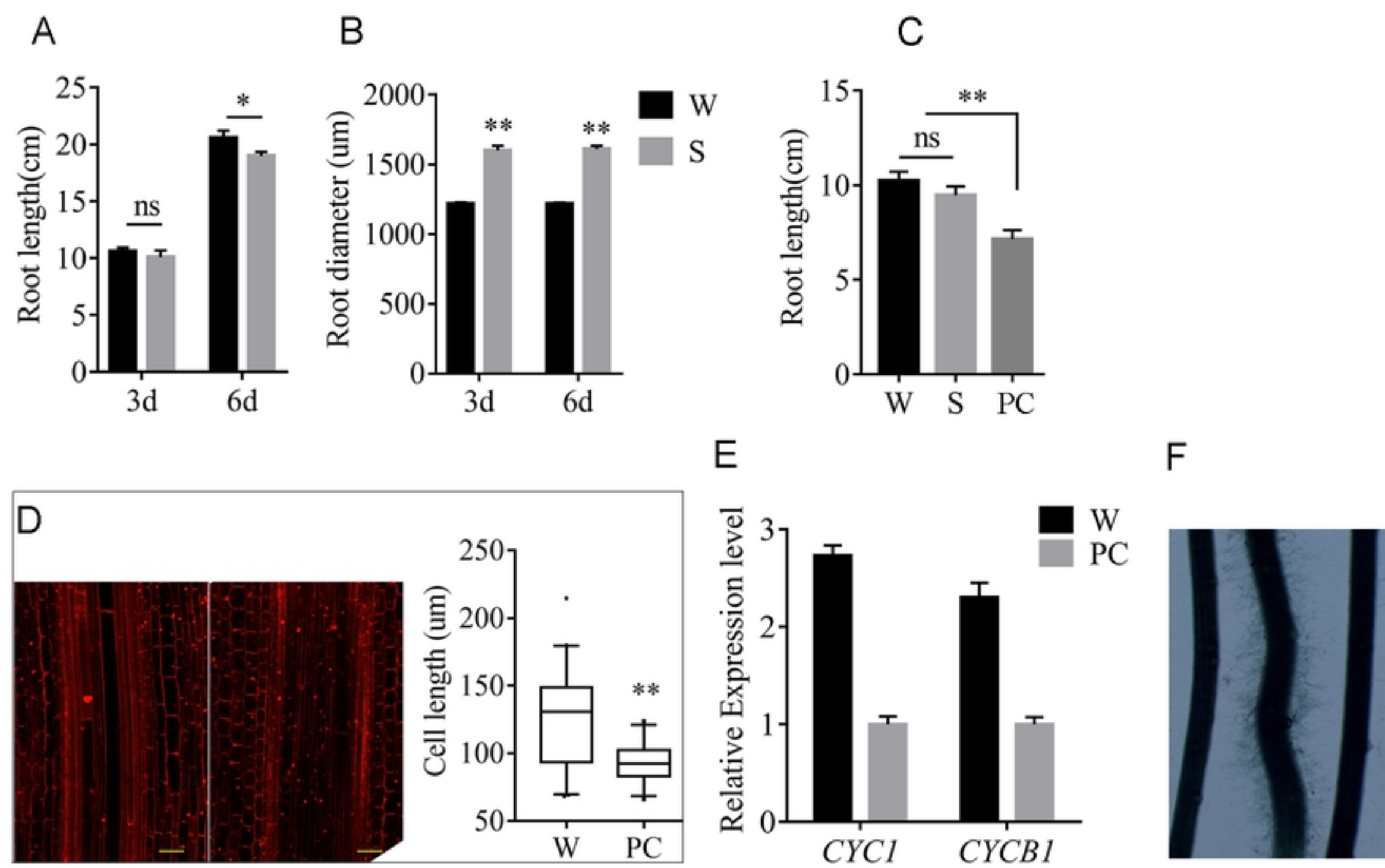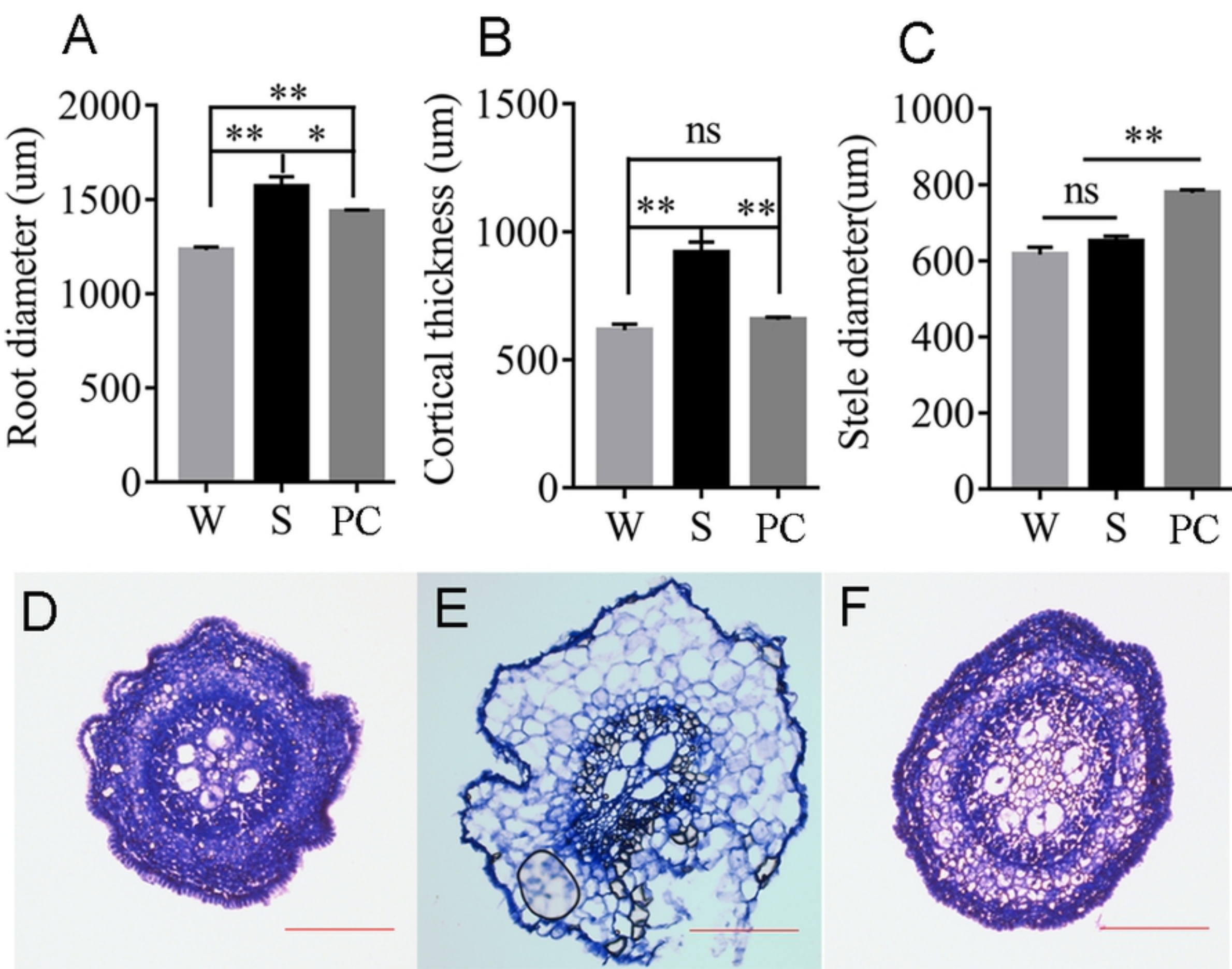314    and SS represent water-, sand- and semi-sand- conditions, respectively.
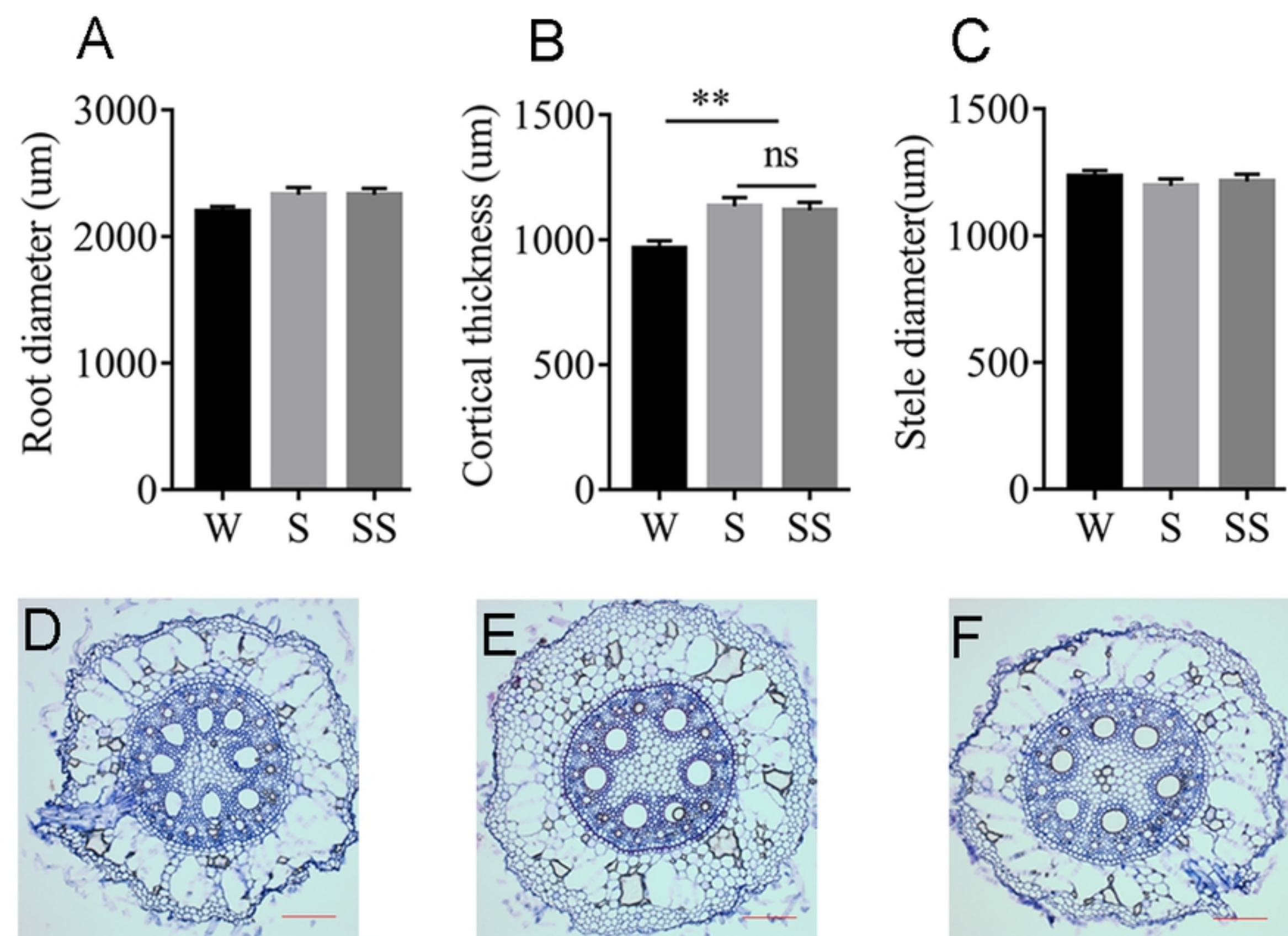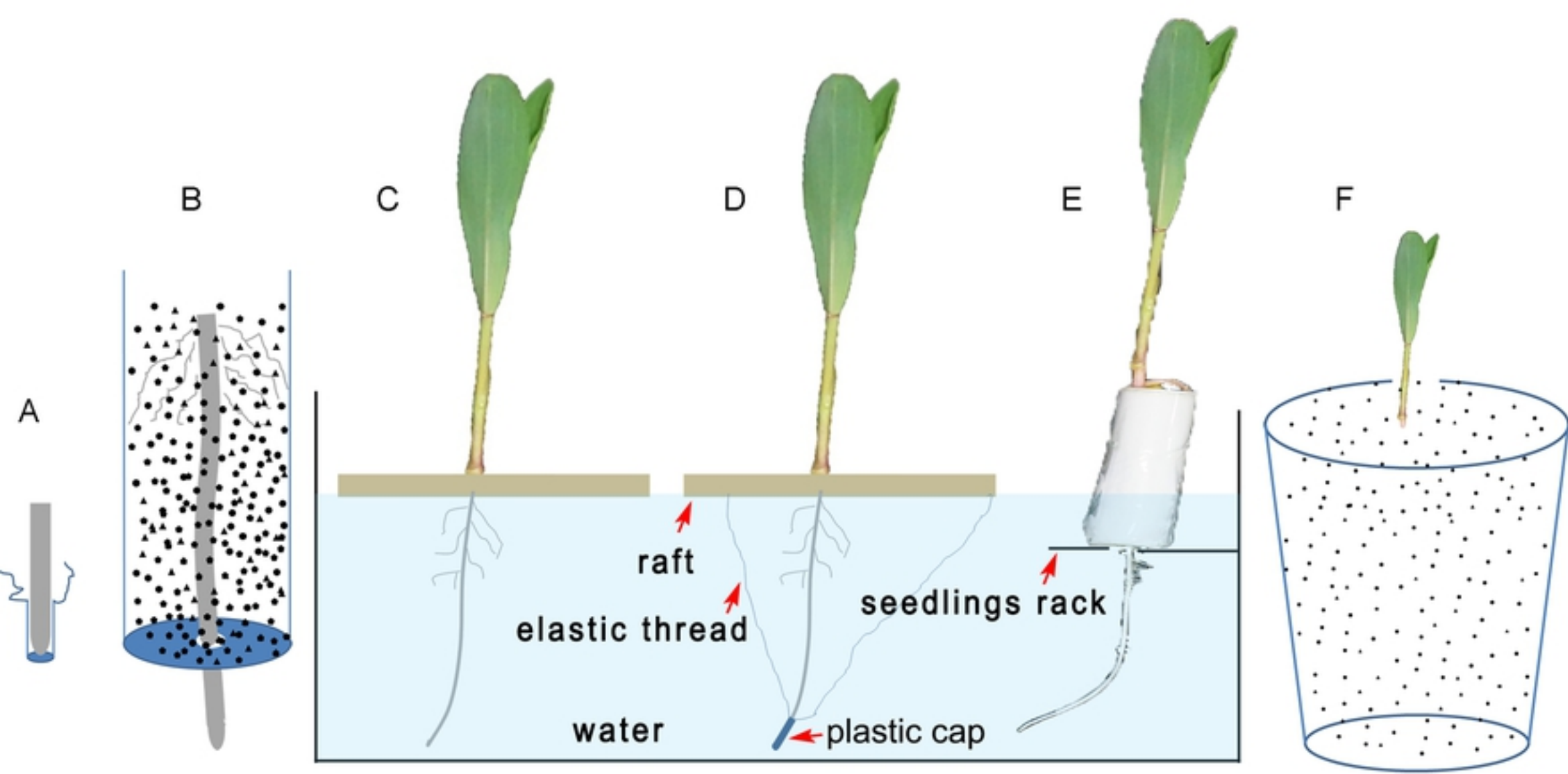
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Fig 2

Fig 3

Fig 4

Fig 1