Morphology of softening and translucency in the stems of tomato and eggplant seedlings due to infection by *Ralstonia pseudosolanacearum* F1C1

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Abstract

Ralstonia pseudosolanacearum F1C1 is a soil-borne phytopathogenic bacterium with a broad host range that infects several economically important crops. This study primarily focuses on the infection of this phytopathogen in two such important crop seedlings: tomato and eggplant. The observations of the study reveal a complex set of symptoms that include drooping and blackening of the seedling stem, as well as blackening, chlorosis, and curling of cotyledon leaves. Notably, the symptom of stem softening and translucency is seen primarily in the water-submerged stem regions of the root-inoculated seedlings. Furthermore, the morphology of stem softening and translucency is more frequent in root-inoculated eggplant seedlings, underscoring a difference between tomato and eggplant seedlings regarding the virulence of the bacterium. By investigating these unique pathological phenotypes in the infected seedlings, this study uncovers unique symptoms previously not reported in seedling inoculation experiments. Such findings deepen our understanding of the complex virulence mechanisms of the pathogen. The work also highlights the distinct escape mechanisms exhibited by seedlings, revealing how some of its host plants might resist wilting despite infection, offering new avenues for future research. These findings contribute to the

understanding of *R. pseudosolanacearum* pathogenesis, shedding light on its virulence and host response mechanisms.

Keywords *Ralstonia pseudosolanacearum*, Tomato, Eggplant, Symptoms, Stem morphology

Ralstonia pseudosolanacearum is a Gram-negative, rod-shaped, soil-borne, phytopathogenic bacterium that significantly impacts economies reliant on agriculture by bringing about wilting in more than 200 plant species belonging to 53 different botanical families (Bhuyan et al. 2023, 2024; Genin 2010). Its Gram-negative nature equips it with elaborate secretion systems essential for establishing pathogenesis. Moreover, its capacity for natural transformation has resulted in the evolution of a genetically diverse array of pathogenic strains. Consequently, this pathogen is part of the larger *R. solanacearum* species complex, which includes phylotypes I-IV that reflect the geographical origin of the strains (Genin 2010). The wide host range, the geographic distribution, systemic infection, the lethal nature of the disease and the occurrence of many potential pathogenicity determinants in its genome stimulate researchers worldwide to study it.

R. pseudosolanacearum enters the host plant through the roots, invades the xylem vessels, spreads throughout the plant, and disrupts the plant's physiology upon reaching a critical concentration, causing wilting and death of the plant (Naik et al. 2023). Considering the systemic nature of its infection, the disease symptoms are complex. In tomato (*Solanum lycopersicum*), wilting initially manifests in the younger, apical leaves at the top of a grown-up plant and gradually progresses downward to the older branches (Din et al. 2016). In contrast, in eggplant (*S. melongena*), wilting follows a bottom-up pattern, beginning with the older leaves and advancing toward the younger leaves (Gousset et al. 2005). In both hosts,

this process eventually leads to the entire plant wilting and collapsing to the ground. While wilting is the most prominent symptom, other manifestations such as chlorosis and stunted growth are among the several disease symptoms that can be observed in its hosts (García et al. 2019). Apart from the well-documented disease phenotypes in grown-up plants observed through soil drenching or stem inoculation methods, we have recently demonstrated *R. pseudosolanacearum* F1C1 pathogenicity in cotyledon stage seedlings of eggplant and tomato using both cotyledon leaf clip and root inoculation methods (Phukan et al. 2019; Singh et al. 2018; Kumar et al. 2017). The pathogenicity in seedlings and the wilting phenotype are similar to grown-up plants considering the virulence factors reported for grown-up plant infection have also been demonstrated to be required for the seedling's infection (Phukan et al. 2019; Singh et al. 2018; Kumar et al. 2017).

The seedling model allows for closely monitoring both the progression of the infection and the disease, providing valuable insights into the pathogen's behavior and effects. As the genome of the phytopathogen harbors numerous potential virulence functions with mechanisms yet to be demonstrated empirically, it is imperative to thoroughly understand various pathogenicity features of *R. pseudosolanacearum* F1C1 in host plants. Since virulence is a multifactorial phenomenon that manifests through various symptoms depending on the interaction with the host plant, using a seedling model becomes particularly useful for large-scale observation of these interactions.

To investigate these pathological dynamics in practice, we conducted experiments that examined *R. pseudosolanacearum* F1C1 interaction in tomato seedlings (variety Pusa Ruby, Durga Seeds, Uttar Pradesh, India) and eggplant seedlings (variety Dev-Kiran (614), Tokita, Karnataka, India) using leaf and root inoculation methods. Upon close observation of the inoculated seedlings, apart from the usual symptom of the drooping of the stem, a plethora of phenotypes were also observed in these tomato and eggplant seedlings under

study. These phenotypes include: (i) blackening of the stem, (ii) softening and translucency of the stem, (iii) emergence of lateral roots, (iv) emergence of true leaves, (v) curling of the cotyledon leaves, (vi) chlorosis of the cotyledon leaves, and (vii) blackening of the clipped cotyledon leaf tip (Fig. 1). It is noteworthy that the emergence of lateral roots occurs above the softened and translucent section of the stem that aids in discarding the primary root attached to the diseased portion, contributing to the plant's effort to escape the disease. Similarly, the emergence of true leaves also aids in escaping disease by discarding the diseased cotyledon leaves. To our knowledge, these phenotypes have not been adequately documented earlier in seedlings infected by *R. pseudosolanacearum*.

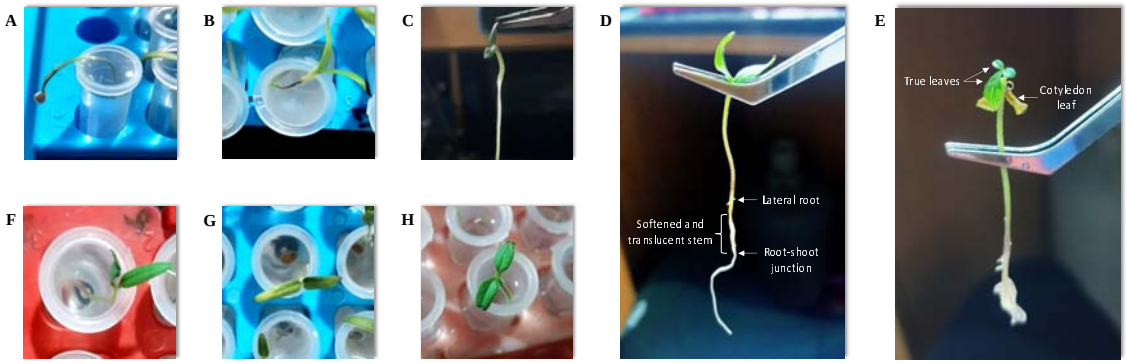

Figure 1. Symptoms of *R. pseudosolanacearum* F1C1 infection in seedlings of tomato and eggplant. **A.** Drooping of the stem. **B.** Blackening of the stem. **C.** Softening and translucency of the stem. **D.** Emergence of lateral roots. **E.** Emergence of true leaves. **F.** Curling of the cotyledon leaves. **G.** Chlorosis of the cotyledon leaves. **H.** Blackening of the clipped cotyledon leaf tip.

Among these above phenotypes, the softening and translucency of the stem are particularly intriguing because the seedlings appeared fully intact with healthy cotyledon leaves. Therefore, this observation was unexpected. This could be observed while inspecting individual plants that appeared healthy, where the increased transparency of the stem region was noticeable. Upon closer examination of the softened and translucent stem under 40X magnification with a compound microscope it was found that the softened and translucent seedling stems had a clear cortex region compared to the stems of an uninfected seedling.

However, the softened and translucent seedling stems appeared to maintain a healthy stele similar to the uninfected ones (Fig. 2). Notably, this phenotype was absent in all control plants that were not inoculated with *R. pseudosolanacearum* F1C1.

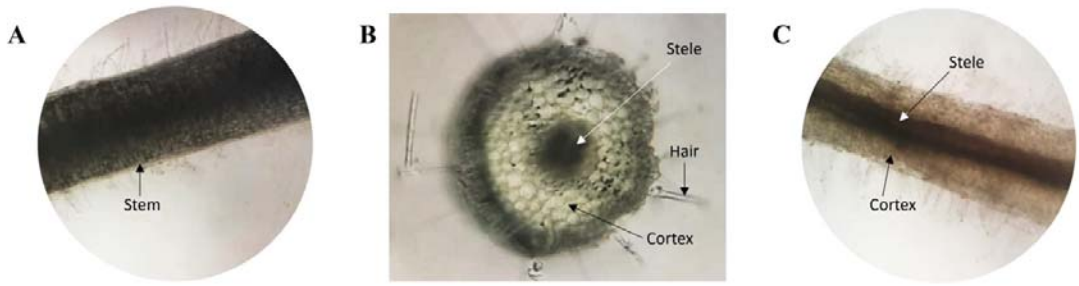

Figure 2. Morphology of softening and translucency of infected seedling stems observed under 40X magnification. **A.** A healthy stem. **B.** T.S. of a healthy stem. **C.** An infected stem.

It is pertinent to note that the morphology of softening and translucency of the stem is predominantly observed in seedlings when *R. pseudosolanacearum* F1C1 is inoculated through the roots rather than in the cotyledon leaves. This softened and translucent stem phenotype is restricted to the stem region that remains submerged in water, which makes it challenging to detect unless the seedling is removed and examined against a light source. The portion of the stem that is above water is visibly affected by the disease, showing signs of blackening (Fig. 3). In a root inoculation, a significantly lesser magnitude of seedling mortality is usually observed in the case of eggplant seedlings in comparison to that in tomato seedlings (data not presented). Also, the softening and translucency of the stem are observed more frequently in apparently healthy eggplant seedlings (≅ 60%) inoculated via the roots than the tomato seedlings (≅ 10%), further demonstrating a difference between tomato and eggplant seedlings regarding the virulence of *R. pseudosolanacearum* F1C1.

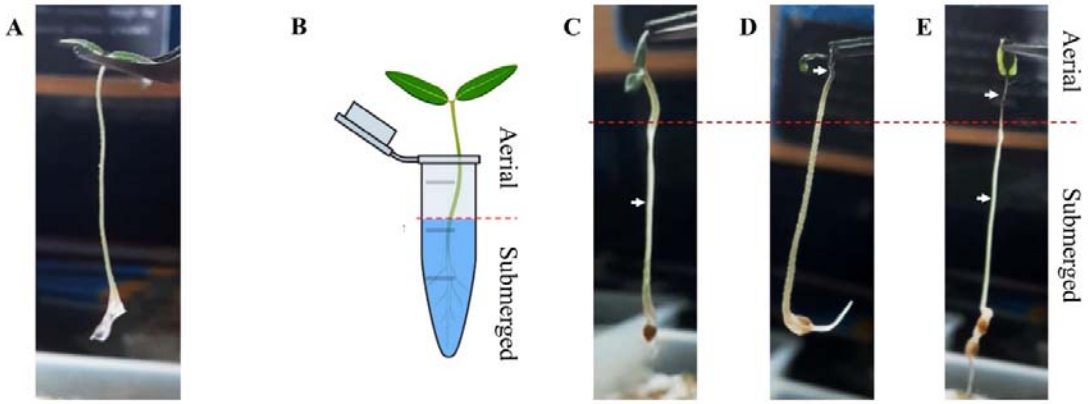

Figure 3. The phenotypes of stem softening and translucency, along with blackening, in *R. pseudosolanacearum* F1C1 infected seedlings. **A.** Uninfected healthy seedling stem. **B.** Graphical representation of the cotyledons' aerial and submerged stem sections, illustrating where the phenotype of blackening and the phenotype of softening and translucency occur, respectively. **C.** The morphology of softening and translucency of the submerged part of the infected seedling stem. **D.** Blackening of the aerial part of the infected seedling stem. **E.** The phenotypes of stem softening and translucency, along with blackening, in a single infected seedling. White arrows indicate the diseased region of the seedlings.

This pathogenic bacterium carries a multitude of virulence determinants such as flagella-dependent swimming motility (Tans-Kersten et al. 2004); type 4 pili-mediated twitching motility (Bhuyan et al. 2024); type II protein secretion system that secretes a consortium of cell wall degrading enzymes such as polygalacturonases, pectin methyl esterases, endoglucanases and cellobiohydrolases (Liu et al. 2005); exopolysaccharides (EPS) (Hayashi et al. 2019b); adhesins such as lectins and filamentous hemagglutinins (Kumar 2014; Kostlánová et al. 2005; Hayashi et al. 2019a); type III secretion system that secretes various type 3 effector proteins to overcome the host plant's defense mechanism (Valls et al. 2006); efflux pumps to efflux out toxic substances (Brown et al. 2007); etc. The expression levels of these virulence factors can vary, potentially leading to different pathological phenotypes in the host. Understanding these pathological phenotypes and the different modes of pathogenesis is crucial, as virulence is a multifactorial phenomenon with various factors

contributing to pathogenicity in different degrees depending on their expression and function within the host plant. Therefore, associating these phenotypes with the magnitude of virulence function expression is essential for unravelling the complexity of *R. pseudosolanacearum* F1C1 infection.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Bhuyan S, Dutta L, Begum S, Giri SJ, Jain M, Mandal M, Ray SK (2024) A study on twitching motility dynamics in Ralstonia solanacearum microcolonies by live imaging. J Basic Microbiol 64:42-49. https://doi.org/10.1002/jobm.202300272
2. Bhuyan S, Yadav M, Giri SJ, Begum S, Das S, Phukan A, Priyadarshani P, Sarkar S, Jayswal A, Kabyashree K, Kumar A, Mandal M, Ray SK (2023) Microliter spotting and micro-colony observation: A rapid and simple approach for counting bacterial colony forming units. J Microbiol Methods 207:106707. https://doi.org/10.1016/j.mimet.2023.106707
3. Brown DG, Swanson JK, Allen C (2007) Two host-induced Ralstonia solanacearum genes, acrA and dinF, encode multidrug efflux pumps and contribute to bacterial wilt virulence. Appl Environ Microbiol 73:2777-2786. https://doi.org/10.1128/AEM.00984-06
4. Din N, Ahmad M, Siddique M, Ali A, Naz I, Ullah N, Ahmad F (2016) Phytobiocidal management of bacterial wilt of tomato caused by Ralstonia solanacearum (Smith) Yabuuchi. Span J Agric Res 14:e1006-e1006. https://dx.doi.org/10.5424/sjar/2016143-9012
5. García RO, Kerns JP, Thiessen L (2019) Ralstonia solanacearum species complex: a quick diagnostic guide. Plant Health Prog 20:7-13. https://doi.org/10.1094/PHP-04-18-0015-DG
6. Genin S (2010) Molecular traits controlling host range and adaptation to plants in Ralstonia solanacearum. New Phytol 187:920-928. https://doi.org/10.1111/j.1469-8137.2010.03397.x
7. Gousset C, Collonnier C, Mulya K, Mariska I, Rotino GL, Besse P, Servaes A, Sihachakr D (2005) Solanum torvum, as a useful source of resistance against bacterial

and fungal diseases for improvement of eggplant (S. melongena L.). Plant Sci 168:319-327. https://doi.org/10.1016/j.plantsci.2004.07.034

8. Hayashi K, Kai K, Mori Y, Ishikawa S, Ujita Y, Ohnishi K, Kiba A, Hikichi Y (2019a) Contribution of a lectin, LecM, to the quorum sensing signalling pathway of Ralstonia solanacearum strain OE1□1. Mol Plant Pathol 20:334-345. https://doi.org/10.1111/mpp.12757

9. Hayashi K, Senuma W, Kai K, Kiba A, Ohnishi K, Hikichi Y (2019b) Major exopolysaccharide, EPS I, is associated with the feedback loop in the quorum sensing of Ralstonia solanacearum strain OE1□1. Mol Plant Pathol 20:1740-1747. https://doi.org/10.1111/mpp.12870

10. Kostlánová N, Mitchell EP, Lortat-Jacob H, Oscarson S, Lahmann M, Gilboa-Garber N, Chambat G, Wimmerová M, Imberty A (2005) The fucose-binding lectin from Ralstonia solanacearum: a new type of β-propeller architecture formed by oligomerization and interacting with fucoside, fucosyllactose, and plant xyloglucan. J Biol Chem 280:27839-27849. https://doi.org/10.1074/jbc.M505184200

11. Kumar R (2014) Studying virulence functions of Ralstonia solanacearum, the causal agent of bacterial wilt in plants. Doctoral dissertation, Department of Molecular Biology and Biotechnology, Tezpur University, Assam. http://agnee.tezu.ernet.in:8082/jspui/handle/1994/473

12. Kumar R, Barman A, Phukan T, Kabyashree K, Singh N, Jha G, Sonti RV, Genin S, Ray SK (2017) Ralstonia solanacearum virulence in tomato seedlings inoculated by leaf clipping. Plant Pathol 66:835-841. https://doi.org/10.1111/ppa.12628

13. Liu H, Zhang S, Schell MA, Denny TP (2005) Pyramiding unmarked deletions in Ralstonia solanacearum shows that secreted proteins in addition to plant cell-wall-

degrading enzymes contribute to virulence. Mol Plant Microbe Interact 18:1296-1305. https://doi.org/10.1094/MPMI-18-1296

14. Naik B, Kumar V, Rizwanuddin S, Chauhan M, Choudhary M, Gupta AK, Kumar P, Kumar V, Saris PE, Rather MA, Bhuyan S, Neog PR, Mishra S, Rustagi S (2023) Genomics, proteomics, and metabolomics approaches to improve abiotic stress tolerance in tomato plant. Int J Mol Sci 24:3025. https://doi.org/10.3390/ijms24033025
15. Phukan T, Kabyashree K, Singh R, Sharma PL, Singh N, Barman A, Jena BR, Ray SK (2019) Ralstonia solanacearum virulence in eggplant seedlings by the leaf-clip inoculation. Phytopathol Res 1:1-11. https://doi.org/10.1186/s42483-019-0030-x
16. Singh N, Phukan T, Sharma PL, Kabyashree K, Barman A, Kumar R, Sonti RV, Genin S, Ray SK (2018) An innovative root inoculation method to study Ralstonia solanacearum pathogenicity in tomato seedlings. Phytopathol 108:436-442. https://doi.org/10.1094/PHYTO-08-17-0291-R
17. Tans-Kersten J, Brown D, Allen C (2004) Swimming motility, a virulence trait of Ralstonia solanacearum, is regulated by FlhDC and the plant host environment. Mol Plant Microbe Interact 17:686-695. https://doi.org/10.1094/MPMI.2004.17.6.686
18. Valls M, Genin S, Boucher C (2006) Integrated regulation of the type III secretion system and other virulence determinants in Ralstonia solanacearum. PLoS Pathog 2:e82. https://doi.org/10.1371/journal.ppat.0020082

Figure legends

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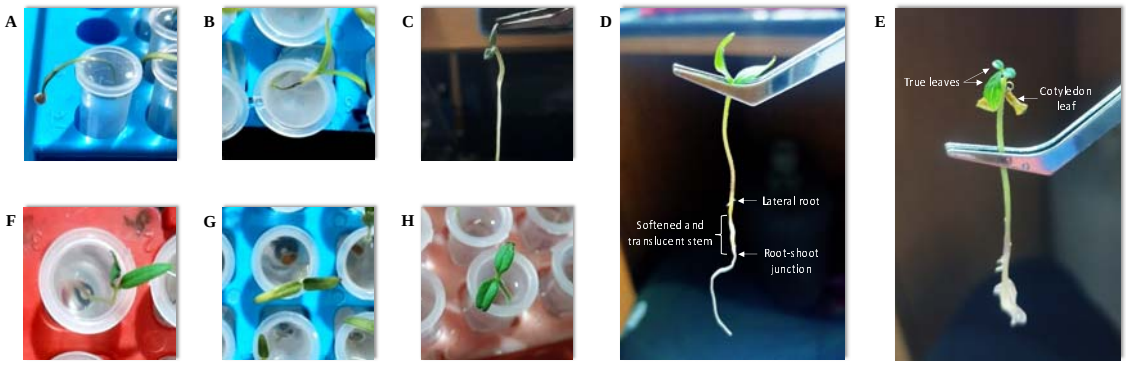
Figures

Figure 1.



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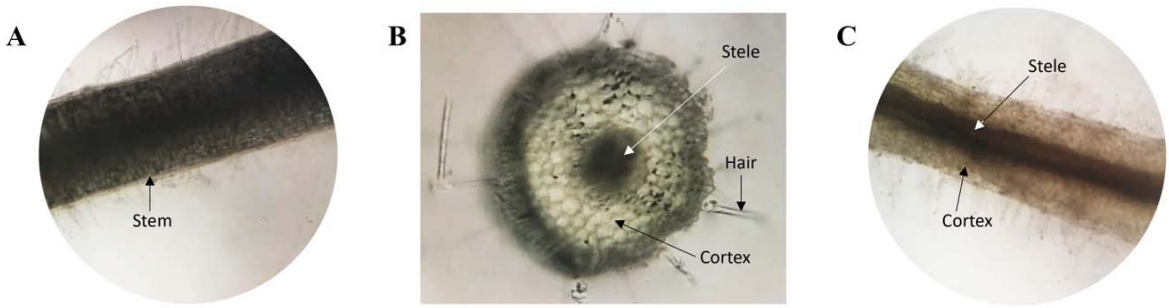

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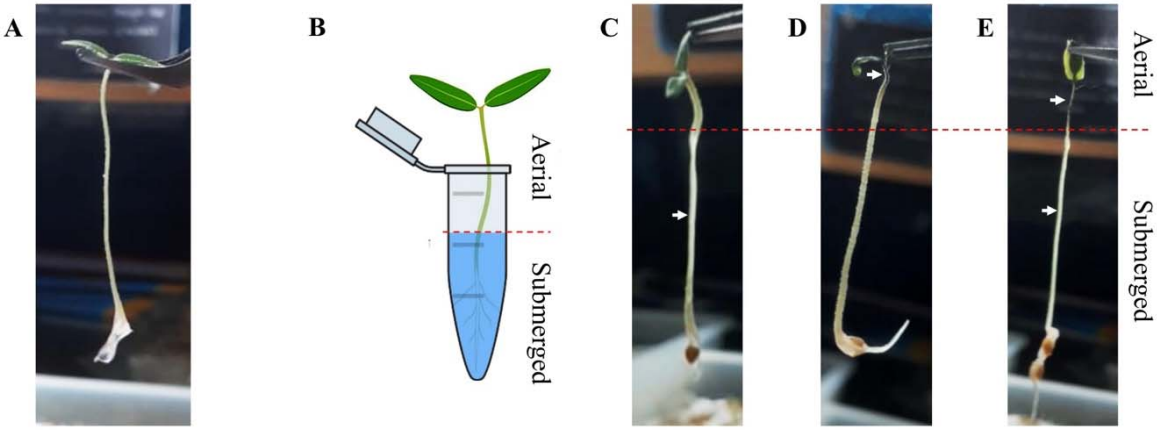

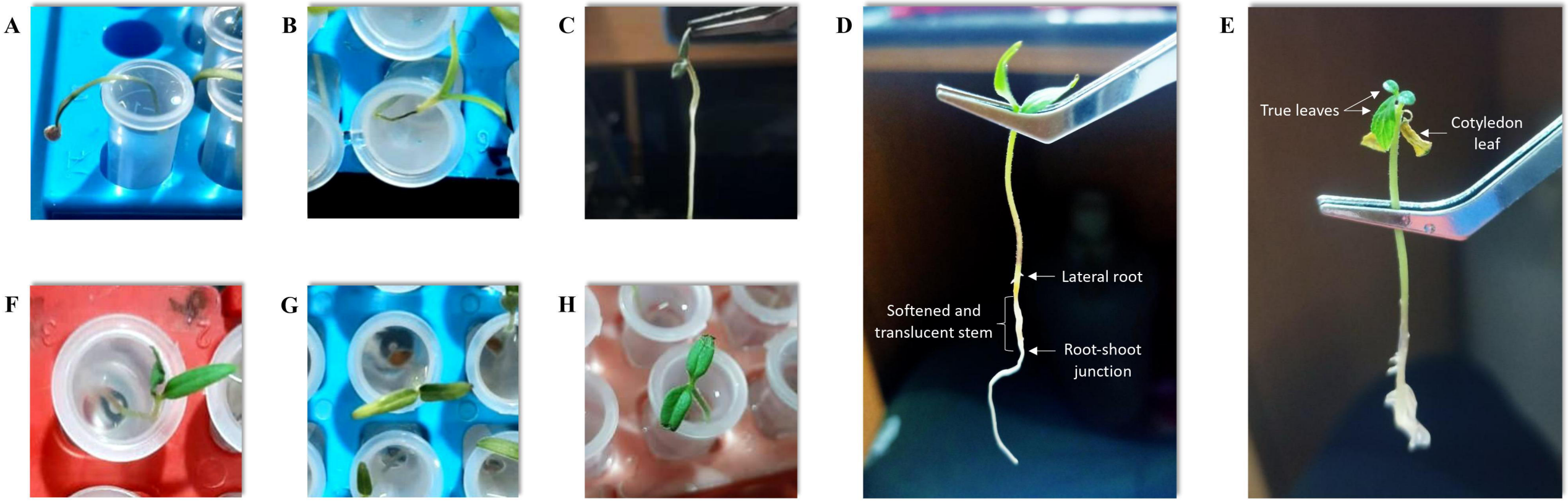
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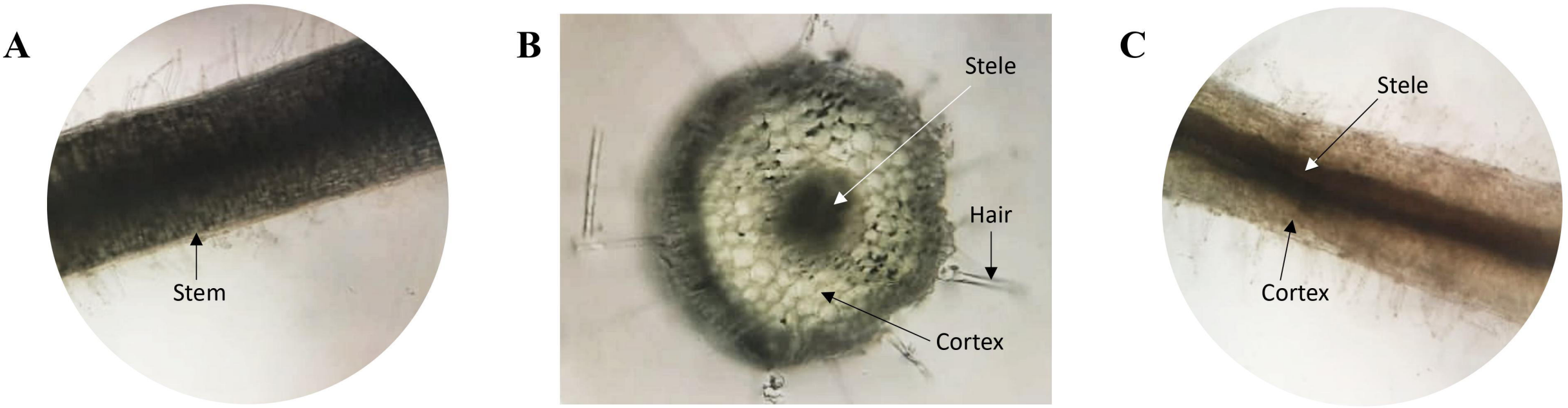
Lateral root
Softened and translucent stem
Root-shoot junction
True leaves
Cotyledon leaf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