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Involvement of Root Hair during *Rhizobial* Invasion in Cultivated Peanut (*Arachis hypogaea* L.)

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Abstract

Peanut root invasion by *Bradyrhizobia* is through a crack entry, which is different from many other legumes applying an infection thread entry in root hair. Understanding the role of root hair in the crack entry of *Bradyrhizobia* invasion of peanut root and subsequent peanut nodulation would facilitate improvement of biological nitrogen fixation in cultivated peanut. The objective of this study was to investigate the involvement of root hair in *Bradyrhizobial* invasion of peanut. Seedling roots of a nodulating peanut cultivar were observed for root hair emergence, its life span, and nodule formation at the base of the lateral roots with and without rhizobia inoculation for 14 days after germination (DAG). Scanning electron microscopy (SEM) was utilized to observe rhizobia accumulation at lateral roots at 24 hours after inoculation (HAI) before the emergence of root hair. Root hair emerged at 7 DAG with or without rhizobia inoculation. Two variations of rosette hair (RoH) were observed, the transient-thin RoH had life span of 3 days after root hair emergence and the thick and densely distributed RoH type stayed till the time of nodule emergence (9 days after inoculation). The lateral root devoid of root hair at the top 2 cm region was found to produce nodules. The SEM observation of seedling roots at 24 HAI showed that *Bradyrhizobia* invaded the roots at epidermis, protoplasm of cortical cell, and cortical cells of the main root near the newly emerged lateral root in the absence of RoH. The observations validated that root hair is not required in the *Bradyrhizobia* invasion of peanut root in the crack entry mode. Results from this study provided important morphological information for the hypothesis of close relationship between

RoH and peanut nodulation for further genetic study of crack entry mechanism and signaling pathway of symbiosis between *Bradyrhizobia* and peanut.

Keywords

Cultivated Peanut, Crack Entry, *Bradyrhizobia*, Root Hair, Nodulation

1. Introduction

Legumes can form a symbiotic relationship with rhizobia to fix nitrogen in root nodules, where the rhizobia reduce atmospheric nitrogen into ammonia (a process known as biological nitrogen fixation-BNF) to supply the hosts' nitrogen need and in return to get nutrients from the host plant. For the symbiotic association, rhizobia must firstly interact with and enter the host root cells. The root epidermis, as a barrier, must be overridden by rhizobia for infection [1]. Different ways of root invasion have been described in the symbiotic relationship of legumes and rhizobia, which is under the genetic control of host plants [2].

The most characterized infection path of rhizobia into host roots is through the intracellular infection thread (IT), which is employed by approximately 75% of legume species including temperate and tropical/subtropical legumes such as *Vicia*, *Pisum Glycine*, and *Phaseolus* [3], and the two extensively studied model legume species, *Medicago truncatula* and *Lotus japonicus*. In the IT mode of invasion, the root hair plays a critical role in assisting *rhizobial* infection. Firstly, the bacteria get attracted by flavonoids or other compounds such as betaines secreted by legume roots. The rhizobia branch on the epidermal cells and attach to the plant root hair, which then causes root hair deformation to form a pocket [4]. The bacteria are then entrapped in the pocket, which further form ITs [5] in the epidermal cells. The rhizobia engulfed in the ITs penetrate the root hair through ITs into the cells, which were inductively formed by actively dividing cortical cells below the site of bacterial infection in the epidermis [6]. Finally, rhizobia are released in the root cortical nodule primordium cells to differentiate into bacteroids. The nodule primordium cells will eventually form nodules for subsequent bacteroids N_2 fixation.

A less studied and alternative mode of infection is intercellular crack entry, which is employed by about 25% of the legume species [7], including *Aeschynomene*, *Stylosanthtes, Sesbania* and *Arachis*. In this type of infection, rhizobia infect the cracks or epidermal cell rupture caused by the lateral root emergence and gain entry to the cortical cells directly through the space between cells [2] [7] [8]. Once rhizobia enter the inner cortex-derived primordial cells, they occupy the intercellular space between epidermal and cortical cells. It was noticed that basal enlarged cells with root hair became the first to be infected by the invading rhizobia cells. Different from the ITs mode of infection, successful infection in crack entry mode of infection is restricted to penetration sites [6]

[7].

Cultivated peanut (*Arachis hypogaea* L.) is a legume crop of immense agricultural and economic importance with numerous uses in several food products. It belongs to the *Fabaceae* family, sub-family *Papilionoideae* [9]. Peanut forms a symbiotic association with rhizobia mainly known as *Bradyrhizobia* through crack entry since ITs have never been seen in the root hair nor epidermal cells, nor developing nodules of peanut [6] [7] [8]. No IT observation during peanut *rhizobial* infection may mean that root hair might not be necessary for peanut root infection by *Bradyrhizobia,* since the involvement of root hair often results into the formation of ITs [10]. *Bradyrhizobia* enter peanut root through an intercellular path, where the epidermal and cortical cells have altered cell walls [11].

Several studies indicated that for intercellular crack entry, rhizobia invade the root at the site of lateral root emergence but none of these studies addressed the role of RoH [11] [12]. However, Chandler [7] stated that infection of peanut only occurs in the presence of root hair and where root hair has large basal cells. The nodulating peanut lines seemed to always have RoH in their lateral root while the non-nodulating lines do not have RoH [5] [13] [14]. For example, Peng *et al.* [15] reported the presence of rosette root hair (RoH) in nodulating peanut plants, but absent in their non-nodulating sister recombinant inbred lines. Further observation of the F_2 and F_3 populations segregating on nodulation showed that RoH was almost completely co-segregating with nodulation. In another word, RoH is only present in nodulating peanut while absent in non-nodulating peanut plants [15]. These observations signal a close relationship between RoH and peanut nodulation, though previous reports indicated that peanut root infection by *Bradyrhizobia* is not through root hair because *Bradyrhizobia* enter the plant at epidermal ruptures [6].

The specific relationship between root hair and peanut nodulation is puzzled. Is RoH needed for *Bradyrhizobia* entry into the root cells of peanut for nodulation process to happen or the genes controlling nodulation in peanut also control RoH development? To provide an initial answer to this question, we investigated the involvement of peanut RoH in peanut root invasion by *Bradyrhizobial* and nodulation. Outcome from this study will clarify whether root hair found at the base of lateral roots are required for *Bradyrhizobial* invasion process of cultivated peanut root, and the association of RoH with nodulation of cultivated peanut. The results will bridge knowledge gaps of genetic and physiological basis of peanut nodulation and contribute to future studies of peanut symbiosis for BNF improvement in peanut and other legume or non-legume species.

2. Materials and Methods

2.1. Plant Materials and Seed Germination

Seeds of Tifrunner, a normal nodulating peanut cultivar [16], were treated with 0.1% $HgCl_2$ solution for 7 minutes with occasional shaking followed by three

times of 5-min rinses with deionized distilled-sterilized water (ddH_2O). The treated seeds were soaked in ddH_2O for 48 hours in growth chamber (Percival CU36:5C8, Perry Iowa) in dark at 25˚C, and then rinsed once and transferred to a 12 × 15-inch germination box with a germination paper (SeedBuro Equipment Company, Des Plaines, IL) absorbed 500 ml ddH_2O. Seeds were allowed to germinate for 5 days in the dark growth chamber at 25˚C until tap roots were about 4 - 6 cm long.

2.2. Rhizobia Infection

Bradyrhizobia strain LB8 [17] was first grown from glycerol stock on YEM plate supplemented with 25 μg/ml chloramphenicol. A single colony from the plate was then grown in YEM broth in 27˚C shaker at 200 rpm until OD 600 = 0.5 - 1.5. Bacteria were precipitated by centrifugation at 1700× g for 10 minutes and washed two times with sterile water. Final pellet was suspended in water and adjusted to OD 600 = 0.12 as inoculum. For inoculation, individual seedling was placed on a petri dish one at a time and 1 ml inoculum of *Bradyrhizobia* train LB8 was applied slowly over the surface of the seedling root, the excess inoculum was blotted dry at the root tip on a sterile filter paper. The whole inoculation process was performed under sterile condition in a certified clean bench. The seedling was then transferred to a 16 oz sterile SteriCon™-8 tissue culture vessel (Phyto Technology Laboratories). The vessel was pre-laid, at the bottom, with two stacks of the germination paper absorbed with 50 ml of 25% Hoagland solution without nitrogen to promote rhizobia infection. The vessel was sealed with lid and incubated in the dark growth chamber at 25˚C for 3 - 4 days until shoots emerged. Then the box was partially wrapped with aluminum foil to cover the roots and was transferred to a light growth chamber with light/dark cycles of 16 h/8 h, 25˚C/27˚C setting. The culture vessels were opened everyday during microscopic observation and pictures were taken using Olympus MVX10 microscope and microscope-coupled Olympus MVX-TVIXC digital camera by using Picture Frame V3.0 software. Cultivated peanut control seedling roots without *Bradyrhizobia* train LB8 inoculation, were also observed for root hair development.

2.3. Observation of Root Hair Appearance and Life Span

At 5 DAG, roots of individual peanut seedlings of both inoculated and non-inoculated groups were observed for appearance of root hair daily for two weeks. Lamp Black (Natural Pigment LLC) water solution was used to mark the secondary roots emerged on 5 DAG or on the day of inoculation or on 0 day after inoculation (DAI). Pictures of peanut roots were taken daily for two weeks using an Olympus MVX-TV1X Cdigital camera and iPAD.

2.4. Tracing Nodule Formation at Non-Root Hair Lateral Roots in Nodulating Peanut Plant

The seedlings of nodulating cultivar Tifrunner were inoculated with *Bradyrhi-*

zobia strain LB8 at 5 DAG. The top 2 cm root segments close to the hypocotyl region, where not every base of lateral root had RoH, were observed for root hair and nodule development for two weeks to investigate if the presence of root hair is required for nodulation in these top 2 cm root segments. Pictures were taken daily until 16 DAI (*i.e.* 21 DAG) using an Olympus MVX-TV1XC digital camera and presence and absence of nodule were recorded.

2.5. Scanning Electron Microscopy (SEM) Observation

Scanning electron microscopy was performed on peanut root as described by Walter *et al.* [18] with modifications. Tifrunner seedling roots at 24 hours after inoculation with *Bradyrhizobia* strain LB8 and prepared for ethanol cryo-fracture SEM. The roots were harvested and immersed into primary fixative containing EM grade, 4% (v/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 (Electron Microscopy Sciences). Sections of the root were razor cut into 10 mm pieces and the region of interest near LR1 and LR2 was lightly pre-scored in the direction of fracture plane. Fixation continued under vacuum infiltration and fixed overnight at 4˚C. The roots were rinsed twice in 0.1 M cacodylate buffer, water washed thrice, dehydrated through a gradient of ethanol (25%, 50%, 75%, 95%, 100%) and placed into a parafilm sleeve containing absolute ethanol. The parafilm sleeve was made by cutting a length twice that of the tissue and wrapped into a tube. One end of the tube was crimped closed and filled with absolute ethanol. While submerged under ethanol, the root section was gently introduced into the tube. The last end of the tube was crimped to seal and immersed into LN_2 until bubbling stopped. The frozen parafilm tube rest on a metal block inside a LN_2 filled Styrofoam box and fractured with a pre-cooled razor blade aligned with the pre-scored lines. The two-halves were collected under LN_2 vapor and immediately placed into pre-cooled absolute ethanol. The fractured root pieces were critical point dried (Bal-Tec CPD-030, Leica Microsystems) and mounted with a carbon adhesive tab and graphite paste onto a 12 mm aluminum SEM stub (Ted Pella, Inc.). The samples were further rendered conductive by sputter coating with Au/Pd and $argon_{(g)}$ (DeskV, Denton Vacuum) and imaged on a Hitachi SU5000 Schottky Field-Emission Variable Pressure SEM (Hitachi High Technologies, America).

2.6. Statistical Analysis

Three regions (0.5 - 1 cm, 1 cm - 2 cm and 2 cm above) of the roots were observed for the occurrence of root hair on their lateral roots for three seedlings (or three replicates). The data were analyzed using analysis of variance (ANOVA) to determine the RoH distribution in different segments of the tap root in Excel. The least significant difference (LSD) test was performed to determine the statistically significant differences of distribution of RoH in the tap root segments. A p value < 0.05 was considered as the threshold for statistical significance between different data points.

3. Results

3.1. Observation of Root Hair Appearance

Roots of individual peanut seedling of both inoculated and non-inoculated were observed daily for the appearance of RoH and the life span of the appeared RoH were monitored daily till they disappeared or senesced. Our observation showed that RoH started to appear at 7 DAG of both inoculated and un-inoculated peanut roots at the base of the lateral roots when the main roots were approximately 6 cm for both transient-thin root hair and densely populated RoH (**Figure 1(b)** and **Figure 2(a)**, respectively). For the individual peanut roots with RoH, we tracked the life span of the observed RoH from the day after root hair emergence (DARHE) to when they senesced (disappeared from lateral root base). Results showed that once the RoH appeared, it usually took approximately 1 - 3 DARHE to senesce for transient-thin root hair (**Figures 1(a)-(f)**). However, RoH could stay on the lateral root up to the time of nodule emergence (*i.e.* 14 DAG) (**Figures 2(a)-(d)**).

3.2. Tracking Root Nodulation at the Non-Root Hair Portion of Nodulating Peanut Plant

Among the observed roots of nodulating plants, we noticed that not every single lateral root had RoHat their base especially at the upper part (0.5 cm - 2 cm) of the tap root close to the hypocotyl region (**Figure 3**). Specifically, no or few RoH were observed at approximately 0.5 cm to the hypocotyl region, while the lower part (>2 cm) towards the root tip of the root system had significantly more RoH (**Figures 3(a)-(e)**). We further tracked lateral roots without RoH for nodule formation with a focus on the lateral roots emerged between 0.5 cm - 2 cm from the hypocotyl region of the main tap root. We observed that a few nodules did form from lateral roots without RoH ever present (**Figures 4(a)-4(o)**).

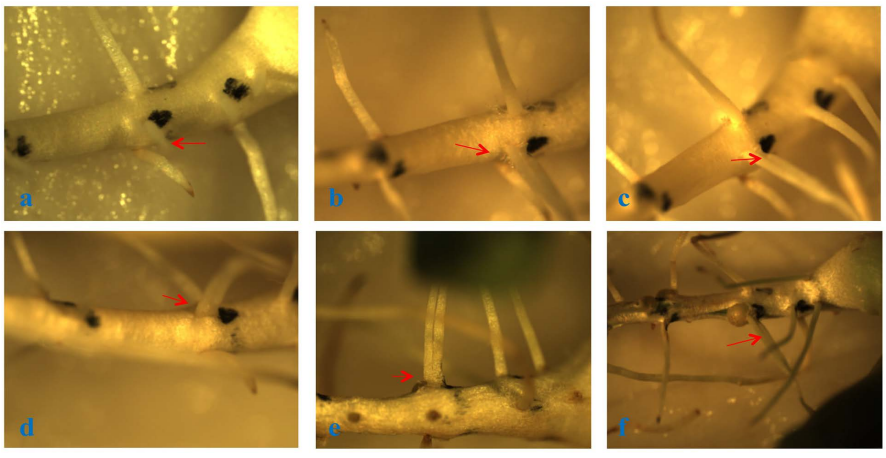

Figure 1. Image showing the emergence and disappearance of transient-thin RoH. (a) Image showing lateral root without RoH at 6 DAG; (b) RoH emerged at 7 DAG; (c) Transient-thin RoH fading away at 8 DAG; (d) RoH no longer present; (e) Nodule starts to emerge; (f) Nodule is fully emerged. * Arrows pointing to the traced lateral root.* Image magnification 10×.

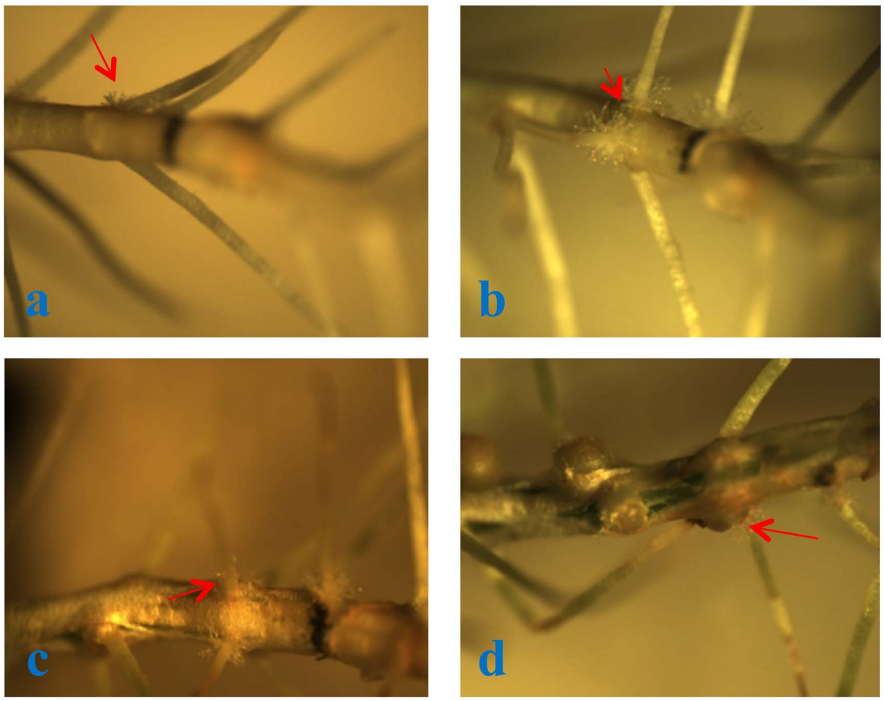

Figure 2. Image showing RoH appearance from the time of root hair emergence to the time of nodule emergence. (a) RoH appeared at 7 DAG; (b) RoH still present at 11 DAG (6 DAI); (c) RoH still present at 12 DAG (7 DAI); (d) RoH present at 14 DAG (9 DAI). *Arrows pointing to presence of RoH. *Image magnification 10×.

3.3. SEM Observation of *Bradyrhizobia* Invasion of Peanut Root before RoH Appear

Peanut root at 5 DAG, before the emergence of RoH, was inoculated with *Bradyrhizobia* strain LB8. At 24 hours after inoculation (HAI), when no RoH (normally appear at 7DAG) was observed yet (**Figure 5**), a cross section of the inoculated peanut root at 0.5 cm - 2 cm top segment of the tap root towards the hypocotyl with lateral roots devoid of root hair was prepared for SEM observation. The observed cross section had two lateral roots with one being emerged after inoculation but before 24 HAI (namely lateral root 1—LR1) and one being emerged before inoculation (namely lateral root 2—LR2). At the bases of both LR 1 and 2, no RoH was observed under SEM. By looking at the base of LR2, no rhizobia accumulation was observed. However, by looking at the base of LR1, impressive patches of rhizobia accumulation were noticed specifically near the surface of a naturally cracked region of the lateral root (**Figure 5(b)** magnified in **Figure 5(c)** and **Figure 5(d)**). A further zoomin, at the surface of LR1 allowed us to observe the accumulation of rhizobia not only on the surface of the epidermal cells but also underneath the biofilm of the root epidermal cells (**Figure 5(d)**) indicating the penetration of rhizobia through the epidermis of the root. We further zoomed into cortical cells of the main root near the LR1. Surprisingly, rhizobia were found to not just have invaded into the intercellular space of cortical cells (**Figure 5(e)**), but also occupied within the root epidermal cells (**Figure 5(f)**).

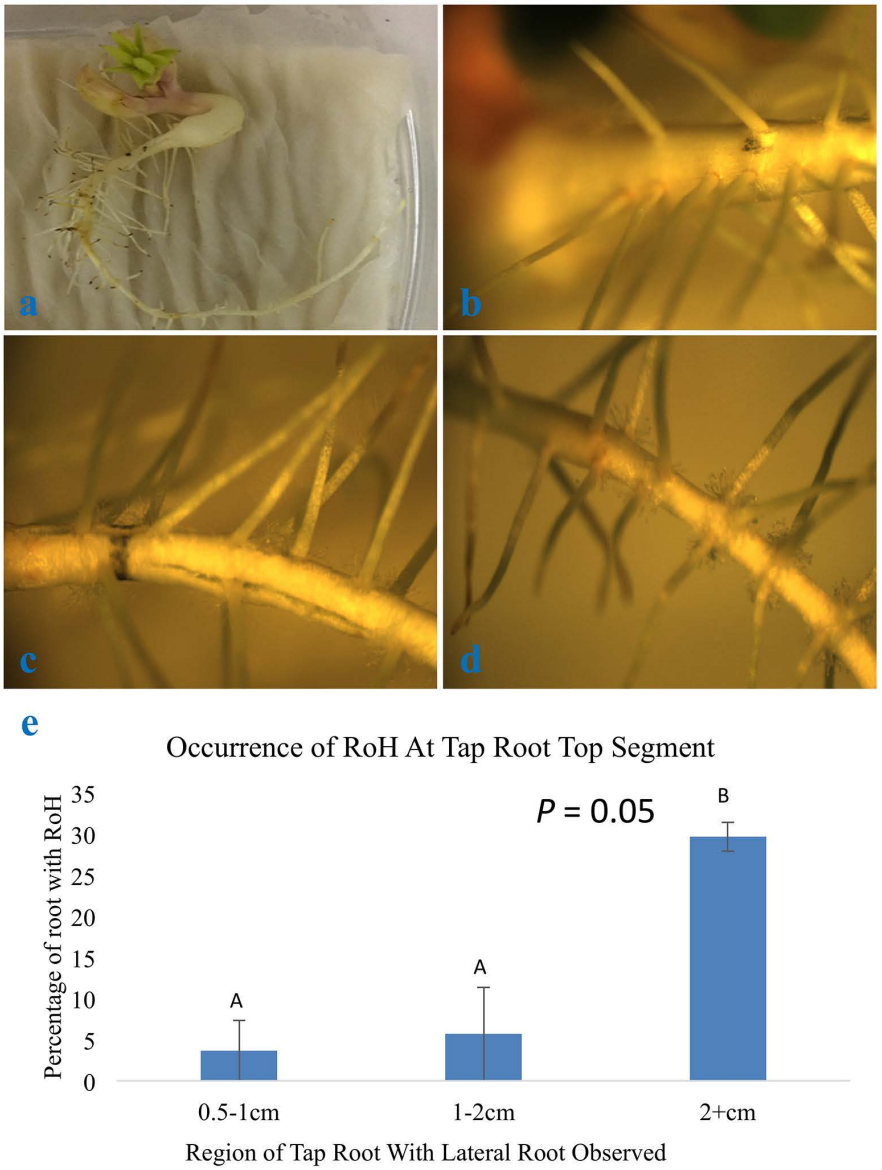

Figure 3. Observation of RoH uneven distribution on peanut root at 9 DAG (4 DAI). (a) Image showing the entire peanut root tracing system used; (b) Image of the top 0.5 cm - 1 cm segment of the tap root with relatively few or no RoH; (c) Image of 1 cm - 2 cm top segment of the tap with relatively more root hair than the 0.5 cm - 1 cm region of the tap root; (d) Image of >2 cm and beyond segment of the tap root (young growing part of root) with virtually all lateral roots having RoH at the base; (e) Graph showing the distribution of RoH. *Arrows showing RoH. *Image magnification 10×.

4. Discussion

The natural capability of legumes to mutualistically associate with rhizobia to convert atmospheric nitrogen into nitrogen nutrition for plants plays a critical role in maintaining the sustainability of agriculture. The notion that different entry routes exist for rhizobia to invade the host and to form functional nodules has attracted the attention of researchers [12]. It has been proposed that intercellular invasion also known as crack entry mode of infection is a more ancient

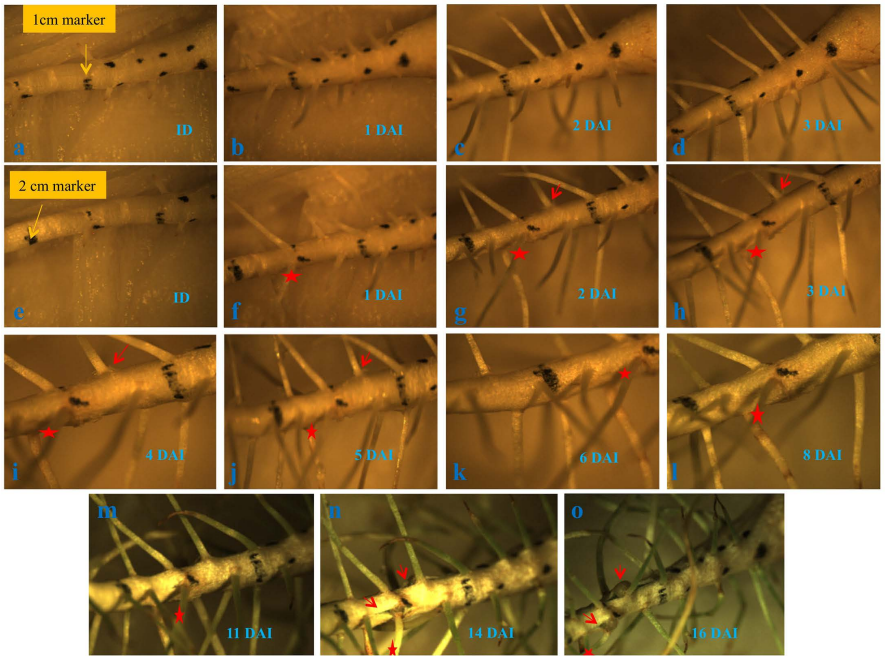

Figure 4. Tracing of Tifrunner (nod+) lateral root devoid of RoH with eventual nodules formation within two weeks of observation. Images of Tifrunner (nod+) tap root with lateral roots (a)-(o). (a) Image showing the 1 cm marked region of the tap root; (e) Image showing the 2 cm marked region of the tap root; (e)-(o) Images showing tap root with a lateral root devoid of RoH; (n) Image showing lateral root devoid of RoH with emerging nodule; (o) Image showing lateral root devoid of RoH with full emerged nodule. *Stars indicate traced lateral root without RoH, arrows are pointing to RoH and nodule. ID means inoculation day. Note: the light color changed due to the use of a new microscope bulb after 8 DAI. *Image magnification 10×.

mechanism than the IT mode of invasion [6] [19]; and a few reviews on legumes symbiosis also indicated that the function of RoH in the crack entry mode of invasion was not clear. Our current study aimed to investigate the involvement of RoH in the crack entry mode of infection in peanut plant. The results shed light on the morphological mechanism of ancient infection path of rhizobia in legume host plant.

4.1. Appearance of RoH

We found that RoH started to appear at the base of lateral roots at 7 DAG with or without rhizobia infection, indicating that RoH is not induced by *Bradyrhizobial* inoculation. However, Wissuwa and Ae [20] observed root hair on 14-day-old peanut seedlings. Likely they only reported when significant root hair was observed since the aim of that particular study was to access root hair on 14-day-old peanut seedlings. Otherwise, the difference in day of root hair emergence could be genotype-or cultivar-specific as different genotypes were used in our study and a previous study [20]. RoH life span varied significantly with some fell off at 2 - 3 DARHE and others staying up till nodule formation, normally at 8 DARHE. The variability of the RoH life span may be due to the nature of the

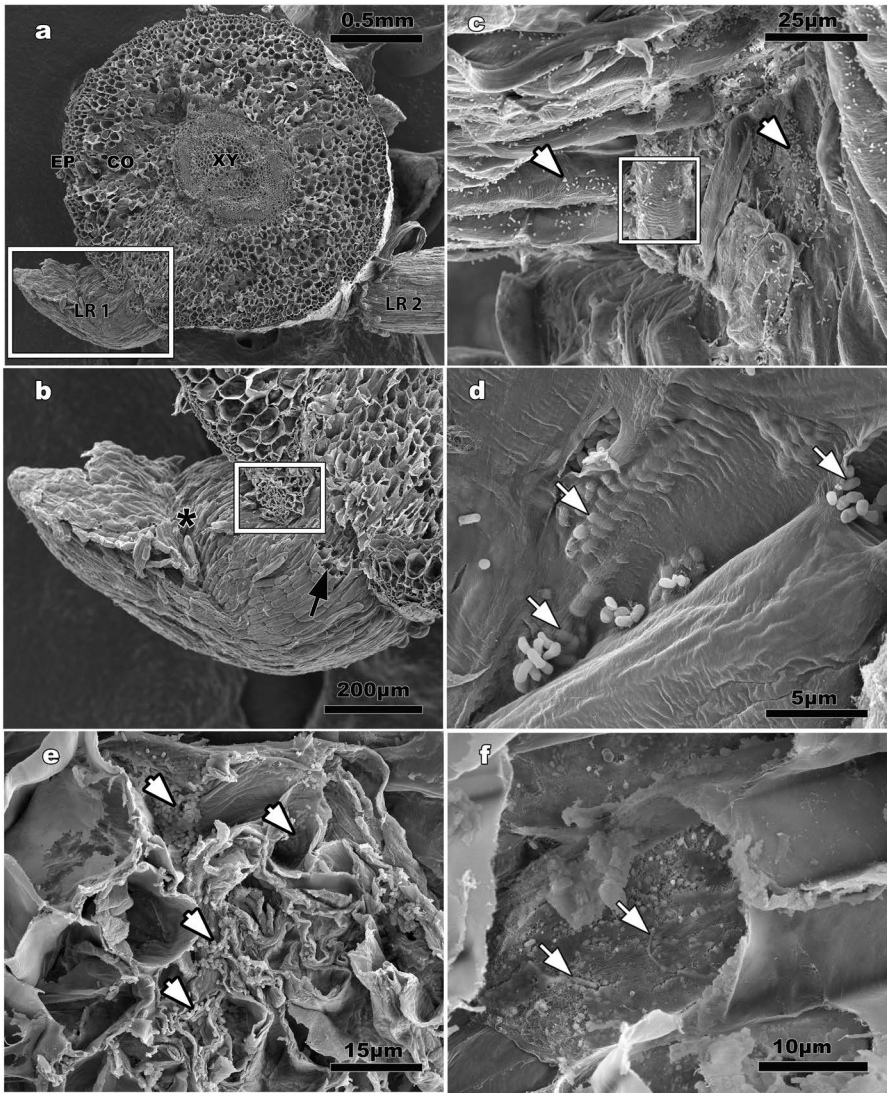

Figure 5. Scanning electron microscopy image of the scanned section of 24 hours *Bradyrhizobia* inoculated root. (a) Low magnification cross-section of primary peanut root structures with lateral roots LR1 and LR2 showing surface of the xylem, cortical, and epidermal cells; (b) Magnified image of (a), showing newly emerged lateral root (LR 1) with epidermal cracks and broken region caused by root emergence; (c) Magnified images of (b*) showing the epidermal cells with *Bradyrizhobia* adhered to surface of cultivated peanut root epidermal cells; (d) Magnified image of (c box), epidermal cells with *Bradyrizhobia* adhered to surface, within cracks and underneath peridermal layers; (e) Magnified image of (b box), fractured cross-section of developing nodule containing microcolony of rhizobia, within nodule cells and interstitial tissue; (f) Magnified image of (b arrow), lateral root epidermal cells fractured to reveal the intracellular structure of cell wall containing rhizobia biofilm. CO, cortex; EP, epidermis; XY, xylem; LR, lateral root; Arrowheads, rhizobia.

RoH formed. The hairs disappeared at 3 DARHE were transiently-thin root hair while the RoH with extended time of stay were typically thick and dense. We also noticed that the dense RoHs were typically present on actively elongating root regions of the seedlings (from 2 cm region of the tap root downward), while

none or few RoHson older root regions (ranging from 0.5 cm - 1 cm top segment of the tap root) during our two-week observation window. This observation is in agreement with previous findings [20], that there were fewer RoH on older roots when compared to the young actively growing roots. The uneven distribution of the RoH on the root may be caused by uneven hormone distribution along the root during elongation, which are regulating the RoH emergence.

4.2. Relationship between Nodulation and RoH

Tifrunner, a normal nodulating peanut cultivar, produces nodules along the roots. However, we noticed that not every lateral root had nodules or RoH at its base, which provided us the opportunity to investigate the relationship between RoH and nodule emergence sites. We observed that there was nodule development from a lateral root base devoid of RoH. This is our first evidence to support that RoH is not required for *Bradyrhizobial* invasion of peanut root and subsequently not required for peanut nodulation, which is in line with previous observation [21] that the formation of peanut root nodule at the base of the lateral roots was a result of proliferating cell divisions derived originally from the pericycle and not necessarily the presence of RoH. We further solidified that RoH is not required for peanut nodulation by SEM observation of the seedlings' lateral roots before RoH emergence. The results further confirmed the invasion of *Bradyrhizobia* in the region of lateral root emergence cells in the absence of RoH at its base.

Karas *et al.* [22] reported an alternative infection mode "crack entry" in *L. japonicus* root hairless 1 mutant, while the wild type of *L. japonicus* is infected through root hair IT infection path. Therefore, they concluded that 'crack entry' mode of infection is used in the absence of root hair in *L. japonicus*. In another word, the root hair is not required for crack entry infection, which is in agreement with our findings, though many other studies indicated that infection of peanut only occurs in the presence of root hair at the base of the lateral roots [7] [13] [14] [15].

Based on these observations, we hypothesized that the genes controlling nodulation most likely also have pleiotropic effect on the development or appearance of RoH. Pleiotropic effect of genes is not rare cases and there are quite a few reports on this effect. For example, three *mlo* powdery mildew resistance genes also have impacts on barley yield [23] [24]. Burstin *et al.* [25] reported that developmental genes have pleiotropic effects on plant morphology and source capacity which eventually have impact on seed protein content and productivity in pea. It has been reported that lateral root emergence, root hair formation, root hair elongation, and nodule development mechanisms have similar phytohormone regulation and auxin being the major positive regulator of these mechanisms [26]. Other hormones such as abscisic acid (ABA), cytokinin, jasmonic acid and so on play opposite roles in these developmental programs/mechanisms [26]. Liang and Harris [27] proposed that the tendency of legumes to form no-

dules may be linked to a difference in ABA sensitivity. Since root hair formation and nodule development mechanisms in different legume species, *M. truncatula* and *L. japonicas,* share some phytohormones [26], it is highly possible that peanut nodule development and root hair formation mechanisms share the same phytohormones, thus the closely linked or co-segregation relationship observed between nodulation and RoH formation in our study and other reported studies [13] [14] [15]. Further genetic and molecular studies on peanut root hair formation and nodulation need to be conducted to test the relationship between RoH, nodulation, their genetic and hormonal controls.

5. Conclusion

Documenting the complete basic information of peanut root, root hair, and nodule formation is very important in studying genetic and molecular mechanisms of peanut nodulation. In this study, we observed the dynamics of RoH and its involvement in *Bradyrhizobial* invasion of cultivated peanut root to investigate its involvement in peanut nodulation. The two weeks observation of cultivated peanut roots at the top segment (0.5 cm - 2 cm) of tap root devoid of RoH, which eventually produced nodules illustrated that RoH is not required for nodulation process and the SEM study corroborated this claim. This conclusion further directs us to the speculation that there are pleiotropic effects of the genetic components controlling peanut nodulation and RoH formation. Since many phytohormones are shared between root hair formation and nodule development in *M. truncatula* and *L. japonicus* especially the positive regulation of auxin in both mechanisms, we hypothesized that these two lateral developmental programs may share similar phytohormones in cultivated peanut. The results resolved the unclear involvement of RoH in crack entry invasion process found in cultivated peanut, which could further guide in the quest of identifying the genes controlling peanut nodulation and eventually in plant nitrogen fixation efficiency improvement.

Conflict of Interests

The authors declare no conflict of interest.

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