Visualization of Peanut Nodules and Seasonal Nodulation Pattern in Different Tillage Systems Using a Minirhizotron System

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ABSTRACT

Nodulation is essential for providing the nitrogen (N) needs of peanut, but little is known about the time course of nodule development with soil depth in a field production system. A minirhizotron system allows for non-destructive, periodic digital imaging of identical locations in the crop root system *in situ*, including the associated nodules. Because the system allows imaging at the same location over time, individual nodule development and subsequent senescence can be followed throughout the growing season. To test the proof of concept for the use of a minirhizotron system to observe peanut nodule development, a case study was conducted in 2012 in Citra, FL in a sod-based production system managed with both conservation and conventional tillage at two different timings. Images were taken to a soil depth of 90 cm on four dates during the growing season, and nodule number, surface area, and senescence were determined. Most nodules occurred at depths spanning 5-30 cm with very few outside of this range; however, individual nodules were noted as deep as 90 cm. In this case study, tillage operation and timing had no impact on the total number of nodules produced, and the peak seasonal nodule number was formed relatively early in the season (9 July – 61 days after planting) and stayed constant until harvest. Nodule number varied by soil depth with the majority of nodules formed in the 0-20 cm depth. Nodule surface area was impacted by tillage type with conservation tillage treatments having larger nodule size (average 2.6 mm^2) than nodules in conventional tillage (1.9 mm^2). Maximum nodule surface area was achieved by mid-season on 1 August. This study gave a unique visual assessment of nodule development for field grown peanut over time and provided data that has rarely been reported. In addition, this study also illustrated that the minirhizotron technique could be successfully utilized in studies examining the development of nodules in peanut and would likely be applicable for similar studies in other legume crops.

Key Words: Minirhizotron, nitrogen fixation, nodulation, tillage, peanut, *Bradyrhizobium*.

The nitrogen fixation process in peanut (*Arachis hypogaea* L.) is essential for providing nitrogen (N) for the crop throughout the season, as it delivers from 50-80% of the total N required by the plant (Boddey *et al.*, 1990). The rates of N fixation for peanut range from 100 to 190 kg N/ha (Boddey *et al.*, 1990), and are quite comparable to other N-fixing crops such as soybean [*Glycine max* (L.) Merr.; 85 – 155 kg N/ha], chickpea [*Cicer arietinum* L.; 103 kg N/ha], or pigeon pea [*Cajanus cajan* (L.) Mills.; 168 – 280 kg N/ha) (Elkan, 1995). The process of biological N fixation (BNF) involves symbiotic bacteria that reduce atmospheric N_2 to ammonia, a form of N that is available for plant nutrition (Nievas *et al.*, 2012). The BNF process can account for as much as 65% of the N that is used in agriculture worldwide, with the largest quantity of fixed N originating from the symbiotic association between rhizobia and legumes (Dupont *et al.*, 2012). For example, intercropped peanut has the capacity to provide over 10% of the total N that is accumulated in the accompanying crop during a growing season (Chu *et al.*, 2004). Therefore, assessing the process of nodulation in peanut could have important implications for evaluating and developing sustainable cropping systems globally.

The process of infection of roots by N fixing bacteria is fairly unique in peanut and occurs through a process known as "crack entry" (Boogerd *et al.*, 1997). The process involves the invasion of rhizobia between the epidermal cells at the base of emerging lateral roots, and the eventual proliferation of bacteria into the root cortical cells (Bogino *et al.*, 2011; Nievas *et al.*, 2012). The majority of nodules in peanut are formed on the first order lateral roots near the basal region (top 8 cm) of the taproot (Tajima *et al.*, 2006). Peanut generally lacks root hairs on the taproot because the surface cell layers are shed, so nodules form at the base of lateral roots where rosette-type root hairs up to 4 mm in length are located (Akasaka *et al.*, 1998). Peanut typically forms determinate type nodules (Akasaka *et al.*, 1998) that are characterized by having no permanent meristem, a regular

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spherical shape, and a homogeneous central tissue within the nodule that is composed of tightly packed bacteroids responsible for the actual N fixation process (Desbrosses and Stougaard, 2011; Dupont *et al.*, 2012). Alternatively, legumes such as pea (*Pisum sativum* L.) or clover (*Trifolium spp.*) form indeterminate nodules that have a permanent meristem and take on a more cylindrical shape (Dupont *et al.*, 2012). The two primary bacterial genera that commonly colonize peanut nodules are *Bradyrhizobium* as well as *Rhizobium* (Nievas *et al.*, 2012; Angelini *et al.*, 2011). However, rhizobia within single peanut nodules can be very diverse with no real dominant strain (Angelini *et al.*, 2011) and often include non-rhizobial endophytes (Ibáñez *et al.*, 2009).

Aside from biological factors such as rhizobial strain or host specificity among crop cultivars, environmental conditions and management practices within typical field production settings can impact the formation of nodules and the subsequent process of N fixation. These factors include: soil temperature; water availability; soil acidity; soil salinity; intercropping; crop rotation; fertilization regime; inoculation practices; and tillage practices (Sprent, 1972; Hungria and Vargas, 2000; van Kessel and Hartley, 2000; El-Akhal *et al.*, 2013). Reduced tillage and cover crops prior to a legume crop often increase nodulation, as is the case for soybean nodulation (Hughes and Herridge, 1989) and rates of N fixation (Kihara *et al.*, 2011) for this crop. Increased N fixation has also been noted under reduced tillage for chickpea and faba bean (*Vicia faba* L.; López-Bellido *et al.*, 2011). One typical system where tillage plays a critical role and impacts the performance of the subsequent peanut crop is bahiagrass (*Paspalum notatum* Flueggé) sod systems. Bahiagrass sod is an important economic component in many Florida peanut production systems either through inclusion of bahiagrass and livestock in rotation with peanut (Katsvairo *et al.*, 2006) or because many producers may rotate long-term bahiagrass fields with peanut to economically benefit their production system. Tillage systems that can be utilized to break up the bahiagrass sod may be limited due to its extensive and fibrous root system. Conventional, deep tillage is primarily chosen, but some growers are considering the economic and environmental benefits of reduced tillage systems (such as strip tillage) that are able to reduce fuel use and protect soil resources. Therefore, the tillage operation chosen to remove the bahiagrass sod could have serious impacts on the nodulation pattern and N fixation of the following peanut crop.

While it is acutely important to assess the impact of tillage and other field management decisions on nodulation and N fixation rates, evaluation and quantification of nodulation patterns in the field is difficult and partially explains the paucity of studies that have successfully achieved these measurements. Methods typically involve the destructive harvest of roots through coring, trenching, or uprooting large quantities of soil – all techniques that are extremely labor intensive, hugely destructive within a research plot area, and most often preclude the ability to perform repeated sampling within a growing season (Gray *et al.*, 2013). Recently however, Gray *et al.* (2013) successfully demonstrated the efficacy of using the minirhizotron technique for the study of nodulation patterns in field-grown soybean under elevated CO_2 combined with drought stress. The minirhizotron technique involves the installation of clear plastic tubes within and parallel to the crop row, usually at a 30 or 40° angle which allows for repeated imaging of the root system through the season (Milchunas, 2012). A camera is inserted within the tube and roots with associated nodules can be imaged along the length of the tube. Images can then be digitally analyzed for nodule number, size, and density as well as other root traits through the use of several software programs. Because the tube normally has a locking mechanism interfaced with the camera, the user can repeatedly image the exact same locations along the length of the tube over time.

The minirhizotron technique may allow for the detailed and comprehensive examination of the natural history of peanut nodule formation by soil depth and across time that has not been previously documented. In addition, the effect of alternative tillage systems on nodulation, particularly those utilized in a bahiagrass sod rotation can also be examined. To investigate the suitability of the minirhizotron imaging technique in determining differences in peanut nodulation, a field experiment was established in Citra, FL using alternative tillage operations in a bahiagrass sod cropping system as a test case. *In situ* nodulation patterns along the root system over the growing season were imaged directly through the use of minirhizotrons.

Materials and Methods

Tillage Treatments and Crop Maintenance

In 2012, field plots were established at the University of Florida's Plant Science Research and Education Unit (PSREU) located near Citra, FL

Table 1. The weeks after planting (WAP) when applications of pesticides and nutrients were made during the experiment at PSREU in 2012.

WAP	Pesticide (L/ha)	Nutrients (kg/ha)
0	1.89 Glyphosate	560 NPK (3-9-18)
	0.95 Pendimethalin	
	0.03 Diclosulam	
3	2.34 S-Metolachlor	
5	1.75 Chlorothalonil	0.03 Boron
6		2240 Gypsum
7	1.75 Imazapic	
	1.75 Chlorothalonil	0.03 Boron
9	0.58 Prothioconazole	
11	1.46 Azoxystrobin	
14	0.58 Prothioconazole	
16	8.76 Pyraclostrobin	
18	1.75 Chlorothalonil	
20	1.75 Chlorothalonil	

(29°24'28'' N, 82°10'30'' W, elevation 21 meters) on a Sparr fine sand (loamy, siliceous, sub active, hyperthermic Grossarenic Paleudults). Plots were arranged in a randomized complete block design with the following treatment factors: three tillage treatments (Conventional, Strip, and Strip followed by use of a High Residue Cultivator – Strip/HRC), and two timings of tillage (all tillage operations one month prior to planting – Date 1, and all tillage at the time of planting – Date 2). The crop followed a well-established stand of bahiagrass. The entire plot area was killed chemically with a 2% (1.9 L/ha) rate of glyphosate. The vegetation in the field was allowed to die down for one month before the early tillage (Date 1) operations. The conventional plots were disked three times, turned with a moldboard plow and smoothed with a disc harrow and field cultivator. The strip tillage plots were stripped using a KMC Rip Strip unit outfitted with subsoil shanks and wavy coulters (Tifton, Ga). For the Strip/HRC treatment, at approximately 50 days after planting the plots were cultivated using a KMC Hi Residue Cultivator (Tifton, Ga).

After completion of the Date 2 tillage operations, all plots were planted on 9 May with the commercial peanut cultivar, Florida-07 (Gorbet and Tillman, 2009) using a Monosem (Edwardsville, KS) air planter with an in-row seed population of 19.7 seed/m. Plots consisted of 8 rows (91 cm spacing) 30.5 m long. At planting, a granular inoculant (Cell-Tech®, Novozymes) was applied to all plots using a hopper box attached to the planter. Supplemental seed was added by hand to row areas across the field that had inadequate emergence and stand populations less than 13.1 seed/m on 22 May. Management of pesticides and fertilization was conducted according to the University of Florida's Institute of Food and Agricultural Sciences (IFAS) recommendations (Table 1).

Measurement of Root System and Nodulation

Nodule establishment and growth were assessed by regularly recording root images through the season in the installed minirhizotron tubes from the soil surface to a depth of approximately 90 cm. To accomplish this, clear plastic minirhizotron tubes (183 cm in length; Bartz Technology, Carpinteria, CA) were inserted in-row and parallel to the crop at a 45° angle with the soil surface in each plot. Roots were digitally imaged on four dates (12 June, 9 July, 1 August, and 17 August) within ordered frames (13 X 18 mm area) along the entire length of the tube using a minirhizotron camera system (Bartz Technology, Carpinteria, CA). The locking mechanism of the camera allowed for repeated imaging of identical locations within the tube at every imaging session. When nodules were present within image frames, depth was calculated and nodules were counted and their size determined using the Image J (National Institutes of Health) software program which calculates the diameter and surface area of each nodule. Nodule density was calculated using the frame area of each image.

Statistical Analysis

Data for total number of nodules and nodule surface area were analyzed using ANOVA in SAS JMP (SAS Institute, Inc., Cary, NC) and Tukey's HSD multiple comparisons test was employed to determine the separation among mean values in these two variables.

Results

Nodule formation was successfully imaged using the minirhizotron technique, allowing for the sequential measurement of the number and size of nodules through the growing season. It was possible to return to the same imaging frame at each sampling date which allowed the phenological study of individual nodules over time. An interesting observation was noted: many nodules were surrounded by air spaces which may be important for adequate gas exchange that must occur during the N fixation process (Figure 1).

Utilizing the minirhizotron system in the bahiagrass sod test case, the pattern of nodulation within the different tillage systems could be assessed with the technique. In this test case, the individual tillage operations had no impact on the total number of nodules at all soil zone depths and image dates (Table 2). However, the date when the tillage operations were performed (approximately 30 days

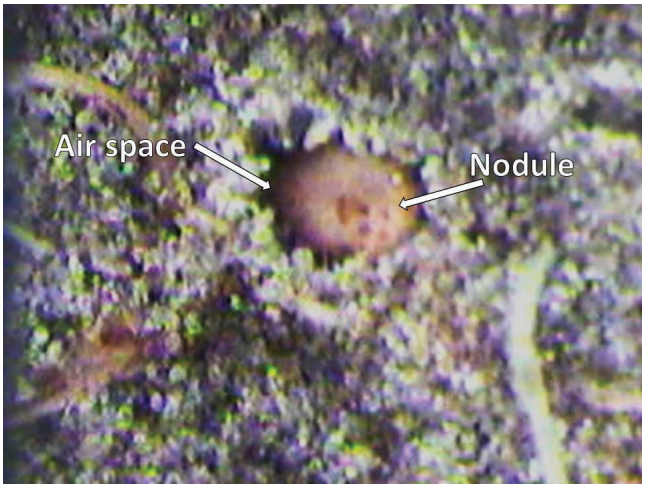

Fig. 1. Observations of air spaces surrounding nodules was common in the current study and may indicate an important factor supporting gas exchange during the N fixation process.

prior to planting – Date 1, or at plant – Date 2) did influence the number of nodules produced, with Date 2 having more nodules than the earlier tillage date. There were no differences in nodule number among the four imaging dates. This indicates that the number of nodules formed was fixed relatively early in crop development, at least by the second image data (9 July, 61 DAP), and stayed constant through the season with few new nodules formed or older nodules deteriorated. This is illustrated in Figures 2 and 3 where groups of nodules became

Table 2. ANOVA results for total number of nodules and nodule surface area as influenced by image date (12 June, 9 July, 1 August, and 17 August), tillage (Conventional, Strip Till, Strip/HRC), tillage date (Date 1, 2), and soil depth zone (0-10, 10-20, 20-30, 30-40, 40-50, 50-60, and 60-70 cm).

		F Ratio	
Factor	df	Nodule number	Nodule surface area
Image Date (ID)	3	2.12	3.13*
Tillage (T)	2	0.76	4.63*
ID*T	6	0.05	0.60
Tillage Date (TD)	1	4.40*	0.06
ID*TD	3	0.03	0.18
T*TD	2	2.12	9.36***
ID*T*TD	6	0.08	0.23
Depth Zone (DZ)	6	11.50***	26.09***
ID*DZ	18	0.16	0.20
T*DZ	12	1.63	4.00***
ID*T*DZ	36	0.10	0.14
TD*DZ	6	1.40	7.73***
ID*TD*DZ	18	0.10	0.14
T*TD*DZ	12	2.61**	1.91*
ID*T*TD*DZ	36	0.10	0.18

*P<0.05
**P<0.01
***P<0.001

apparent by 9 July (61 DAP) and persisted until the last image date on 17 August (100 DAP). This indicates that nodules are fully formed by the crop's peak pod fill (60 DAP) and persist through

Fig. 2. Sequential images of a peanut root system illustrating nodule development and possible senescence within the study site taken on: 12 June (a), 9 July (b), 1 August (c), and 17 August (d).

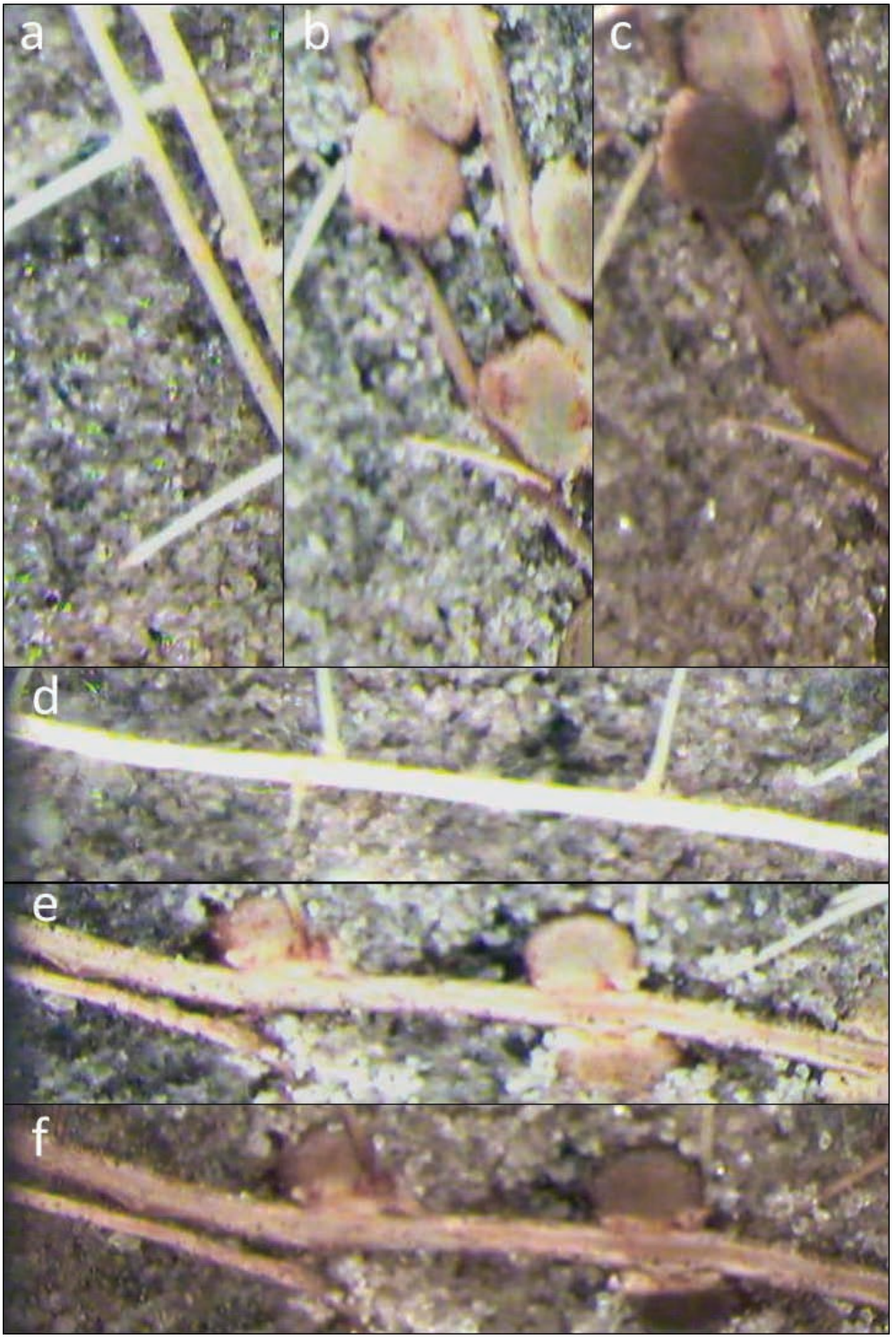

Fig. 3. Sequential development of nodules over time on two root segments illustrating nodule establishment and possible senescence. Images taken on 12 June (a,d), 1 August (b,e), and 17 August (c, f); note that the images taken on 9 July were identical in nodule size and color as the images from 1 August, but due to a preferred background color, the 1 August images are shown.

peak physiological performance (90 DAP). There was one instance where nodules were noted to have senesced and disappeared (Figure 4), but based on the analysis results, this was a fairly rare occurrence. Nodule number differed among soil depth zones (Table 2), with the majority of the nodules formed within soil depth zones 1 and 2 (0-20 cm in depth, Figure 5). The deepest single nodule in the study was noted at 90 cm below the soil surface in the Strip/HRC treatment and

Fig. 4. Root segment image illustrating the process of nodule senescence and eventual deterioration: nodules are visible on root segment 9 July (a) but are absent on 1 August (b).

persisted through to the 17 August imaging date (Figure 6).

To illustrate general nodule density and the pattern of density development by depth across the primary root area examined, nodule density was calculated as the number of nodules within the reading frame area (13 X 18 mm = 234 mm^2 area) and graphed along the rooting depth imaged (roughly 90 cm below the soil surface). Figure 7 illustrates the general pattern of nodule density by soil depth across all tillage and tillage date treatments for three imaging dates. This illustrates how nodules were clustered within the top 20 cm of soil and that the nodule density became fairly constant by 9 July. Nodule density below 40 cm was only noted for 9 July and 17 August, likely reflecting the overall pattern of root extension to deeper soil depths by 9 July.

Nodule size expressed as surface area differed among tillage treatments with Strip and Strip/HRC both having larger nodules than Conventional tillage (Table 2). This was the case regardless of

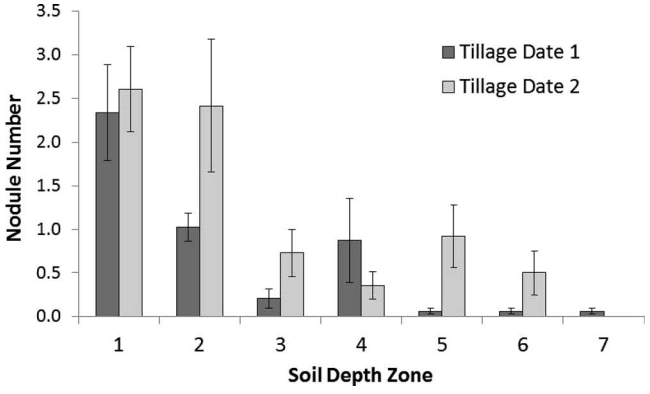

Fig. 5. Average number of nodules by soil depth zone across all tillage treatments but separated by the date of the tillage operation (Tillage Date 1 – one month prior to planting; Tillage Date 2 – at planting). Zones are depths from the soil surface: 1 (0-10 cm), 2 (10-20 cm), 3 (20-30 cm), 4 (30-40 cm), 5 (40-50 cm), 6 (50-60 cm), and 7 (60-70 cm).

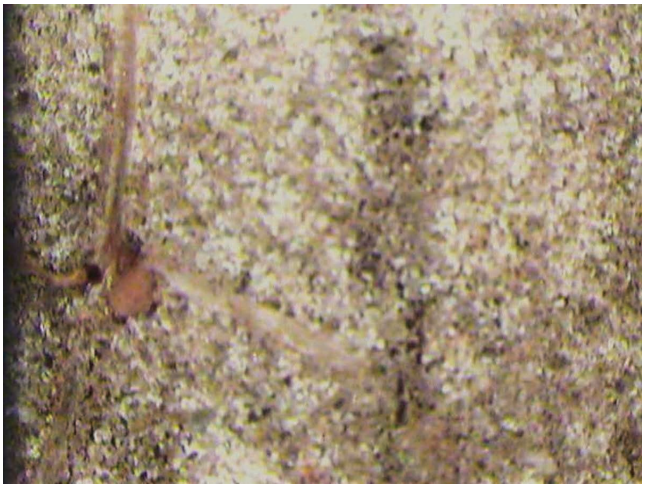

Fig. 6. The deepest single nodule in the 2012 study was located at 90 cm below the soil surface. The nodule was located in the Strip/HRC treatment and persisted until the last imaging date (17 August).

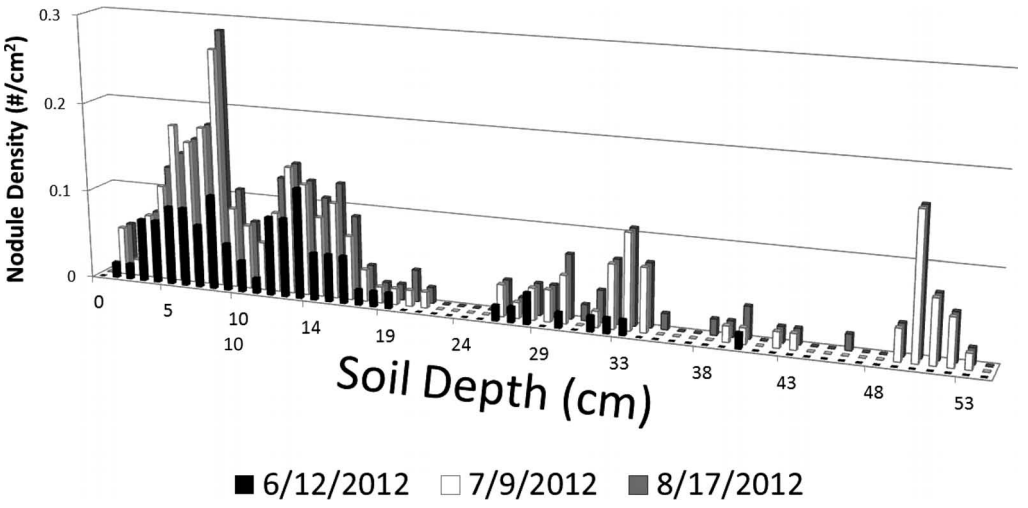

Fig. 7. Average nodule density (# nodule per soil area within reading frame – 13 X18 mm) by depth. Patterns of nodule density are shown for three imaging dates 12 June, 9 July, and 17 August.

when the tillage operation was performed (Date 1 or 2) resulting in average nodule surface areas of 1.9, 2.7, and 2.5 mm^2 for Conventional, Strip, and Strip/HRC, respectively (Figure 8). There were differences among the image dates with the maximum nodule surface area being reached on 1 August; the surface area values at this date were significantly different than the first image date on 12 June. Visually, the expansion of nodule size is illustrated by images in Figure 9 where the enlargement of a single nodule is shown. Nodule surface areas were different among soil depth zones with larger nodules congregated in the top 20 cm (Figure 10). There was an interaction between tillage and depth zone (Table 2) driven by the trend of larger nodules in the Strip/HRC in zone 2 more so than the other two tillage treatments (Figure 10).

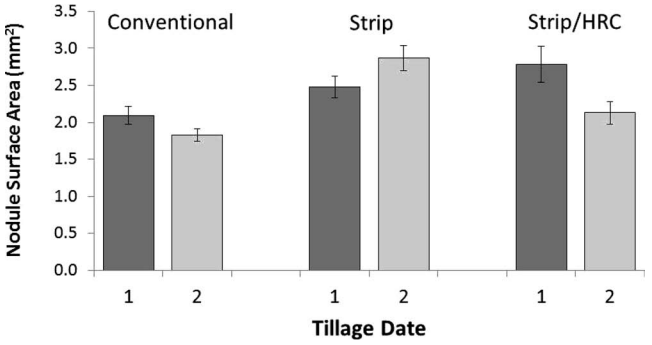

Fig. 8. Average individual nodule surface area (mm^2) across all soil depths and imaging dates. Results are shown for three tillage treatments (Conventional, Strip, and Strip/HRC) and the dates of tillage operation (1 month prior to planting – Date 1, and at planting – Date 2).

Discussion

There are several important contributions from this study: 1) the general observational and quantitative assessment of the natural history and progression of peanut nodule formation, both temporally and spatially; 2) the evaluation of the minirhizotron system in a test case examining the impact of tillage on this natural history; and 3) the demonstration of the distinctive and expansive research applications that the minirhizotron technology can afford when studying belowground processes. In general for most crop species, the pattern of root nodule formation in relation to different soil depth zones has rarely been reported (Tajima *et al.*, 2006). The minirhizotron system is perfectly suited for this type of study, but this technique has not been commonly utilized to date for studying nodulation patterns (but see Gray *et al.*, 2013), and the current study is certainly the first for peanut.

Based on the current results, the nodulation pattern over time indicated that nodules were formed relatively early in the growing season and the number was set by 9 July (61 DAP) corresponding to peak pod fill (Boote, 1982). New nodule formation or nodule senescence past that date was relatively rare. This pattern may be due to the suppressive ability of early-formed nodules on subsequent nodule development (Kossiak and Bohlool, 1984; Danso and Bowen, 1989). This timing of nodule production for peanut is similar to studies on other species noting that primary nodulation is attained by crop flowering (Voisin *et al.*, 2003). As far as nodule pattern within the root system, the current study showed most nodules were formed within the top 20 cm below the soil surface, a pattern that is also similar to

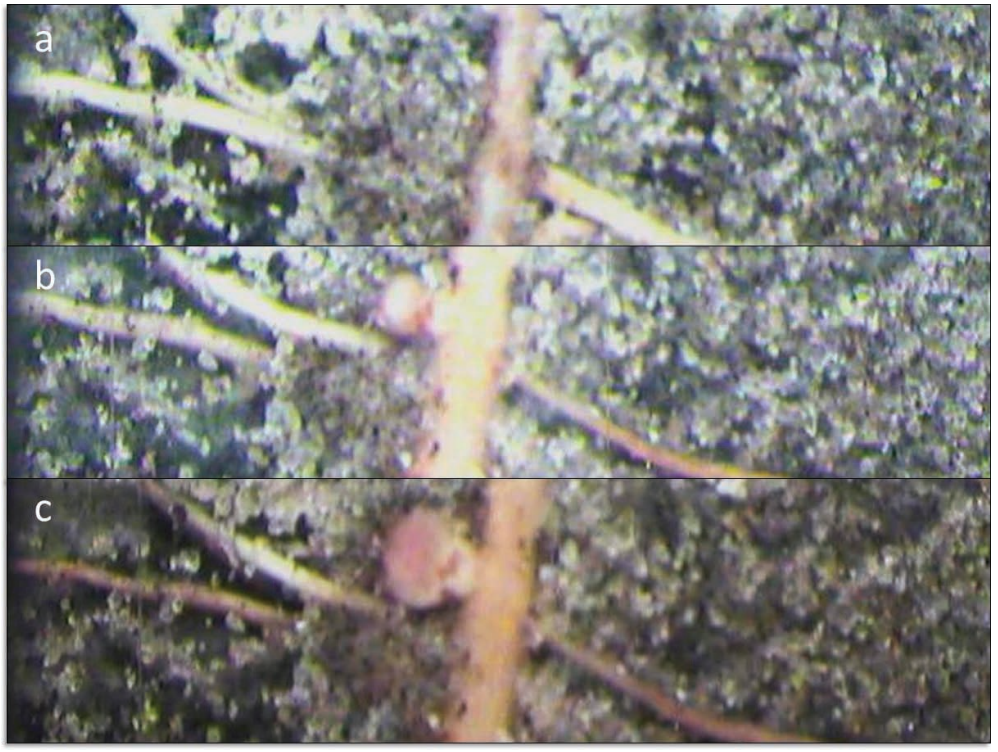

Fig. 9. Illustration of nodule increase in girth over time. Images were taken 12 June, 1 August, and 17 August.

other studies (Danso and Bowen, 1989; Hardarson *et al.*, 1989; Tajima *et al.*, 2006). However, the current study did note an increase in nodule surface area over time indicating that, while nodule number may have been set early in crop development, nodule expansion was certainly possible through the season.

The variability among different soil depth zones in the nodule density and size found in this study has important implications regarding the rhizobial population that was likely infecting the different root segments. Native rhizobial populations are normally widely distributed throughout the soil profile (Bogino *et al.*, 2011). This is unlike the patterns of distribution for rhizobial strains used in commercial inoculants applied in-furrow or directly on the seed that normally remain near the soil surface (0-10 cm); this is because movement of these rhizobial strains through the soil profile is very slow (Hardarson *et al.*, 1989; McDermott and Graham, 1989; Bogino *et al.*, 2011). Native rhizobial populations are also highly competitive

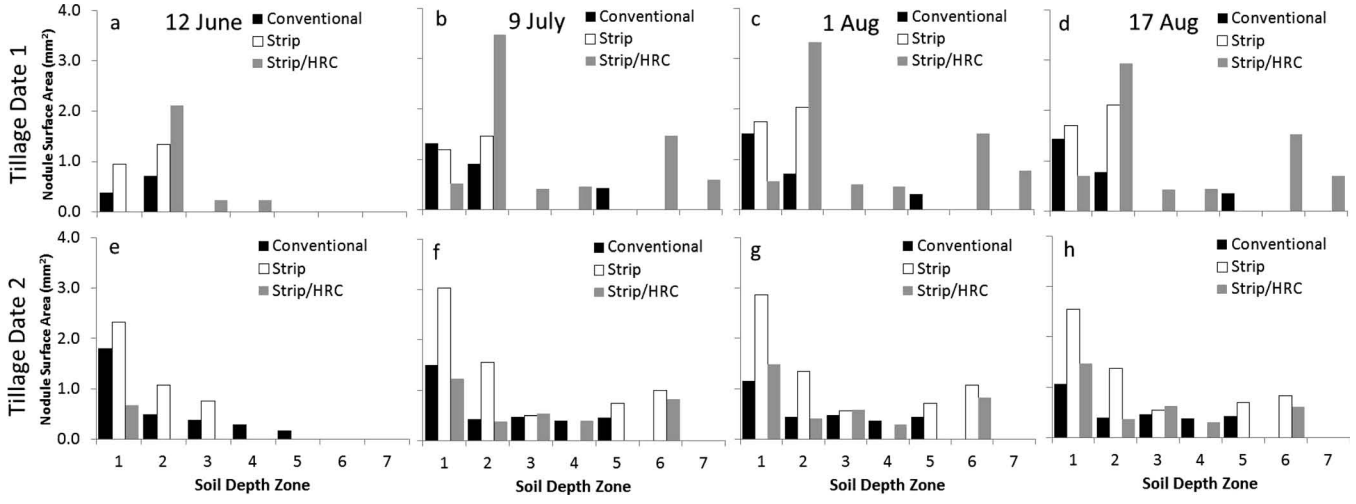

Fig. 10. Nodule surface area by soil depth zone. Zones are depths from the soil surface: 1 (0-10 cm), 2 (10-20 cm), 3 (20-30 cm), 4 (30-40 cm), 5 (40-50 cm), 6 (50-60 cm), and 7 (60-70 cm). Treatments include Tillage Date 1 (a-d) and Tillage Date 2 (e-f); within each tillage date, four imaging dates are included: 12 June (a,e), 9 July (b,f), 1 August (c,g) and 17 August (d,h).

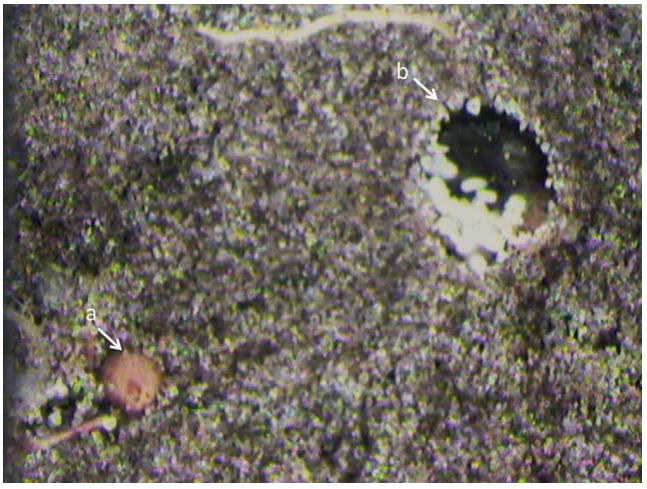

Fig. 11. Image of nodule (a) and possible arthropod egg masses (b) within a soil cavity; image taken on 12 June.

against commercial strains and will normally interfere with the effectiveness of infection by these applied inoculants (Bogino *et al.*, 2011). In the current study, the zones where nodule density and size were high occurred in two zones, 0-10 cm and 10-20 cm. This indicates that perhaps the applied inoculant in the 0-10 cm and native rhizobial strains in the 10-20 cm and deeper soil zones were both active and effective. This is strictly speculative, but testing to confirm and quantify the contribution of native rhizobia seems important in evaluating the effectiveness of commercially applied inoculant. The soil depth zone position of nodules is also important because it is known to directly impact N fixation activity (Hardarson *et al.*, 1989). Roots near the surface derive a higher proportion of their N from the symbiosis with rhizobia (Lory *et al.*, 1992) while deeper nodules have relatively low levels of N-fixation (McDermott and Graham, 1989; Tajima *et al.*, 2006), sometimes due to inhibitory levels of incorporated N fertilizer. However, conservation tillage systems that provide limited soil mixing may have lower nutrient levels at deeper depths which may actually help increase the N-fixing activity of nodules within these regions because the nutrient concentrations would rarely reach inhibitory levels (Virginia *et al.*, 1986).

Nodule size is known to have a direct impact on N fixation (Voisin *et al.*, 2003; Tajima *et al.*, 2007) particularly under stress conditions (King and Purcell, 2001). Large nodules may have amplified N fixation activity in comparison to small nodules due to a greater phloem supply (Purcell *et al.*, 1997). It has been estimated for soybean that, by volume, 25% of a 2 mm diameter nodule is infected, while a 4 mm diameter nodule has 60% infected tissue indicating greater relative N fixation ability (King and Purcell, 2001). In general, peanut has smaller nodules than soybean, but higher overall N fixing activity (Tajima *et al.*, 2007). This size range was similar to that found in the current study where most peanut nodules were approximately 2 mm^2 in surface area, translating to a 1.4 mm diameter.

This case study was successful in evaluating the efficacy of the minirhizotron system for evaluating the impact of tillage practices on the natural history of nodulation including responses in nodule number and size. Previous studies have found a promotion of nodulation under no-tillage systems and that there was a close positive relationship between nodule number and the N-content of roots within conservation tillage systems (Hughes and Herridge, 1989; Sidiras *et al.*, 1999; Kihara *et al.*, 2011; López-Bellido *et al.*, 2011). Tillage system in the current case study had no effect on nodule number but did significantly influence nodule surface area (a surrogate for overall nodule size). The current results indicate that nodule surface area was enhanced in both conservation tillage treatments (Strip and Strip/HRC), regardless of whether the tillage operation was performed one month prior or at planting. Because of the strong relationship between nodule size and N-fixation rates (Purcell *et al.*, 1997; King and Purcell, 2001; Voisin *et al.*, 2003; Tajima *et al.*, 2007), this result indicates a possible advantage of conservation tillage systems through enhancement of N-fixation rates as a whole. However, because the data was relatively limited in scope and primarily aimed at evaluating the use of the minirhizotron technique in different tillage systems, these results require further study and confirmation.

Another contribution from this study is the illustration of the application of the minirhizotron technique in a myriad of possible studies focused on belowground processes. Aside from root measurements and nodulation patterns, the minirhizotron images captured in this study illustrate additional assessments of belowground community dynamics that could be possible using the technique. Other soil organisms were captured in the images from the current study including arthropod egg masses (Figure 11). Studies concentrating on populations of soil organisms could also be digitally imaged using this technique. In the particular case study, further examination of the interaction between the endophytic fungi of bahiagrass and rhizobia on subsequent colonization in peanut could also be studied using minirhizotrons. The current study and similar applications of the minirhizotron technique show the potential of this method to provide novel and important imaging of phenomena that have previously been

unobservable. This was certainly the case for the current study's documentation of nodulation patterns and processes and opens the door for additional unique investigations.

Acknowledgments

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