

Developmental Regulation of Nodulation in *Arachis hypogea* (peanut) and *Aeschynomene americana* (jointvetch)

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Abstract

Legumes in the tribe *Aeschynomeneae* develop nodules only at the site of lateral root emergence. Rhizobia infect the host through epidermal cracks rather than utilizing infection threads in root hairs. In this study we have examined the ontological factors that regulate nodule distribution on two members of the tribe, jointvetch (*Aeschynomene americana* L.) and peanut (*Arachis hypogea* L.). In both hosts nodulation is generally restricted to young lateral roots, ≤ 2.5 cm in length at the time of inoculation. This developmental window is independent of concomitant maturation of the tap root. In peanut, removal of all visible lateral roots from mature tap roots caused new laterals to emerge; these lateral roots nodulated at a frequency similar to that of young laterals on untreated tap roots. Additionally, removal of lateral root meristems two days after inoculation did not alter nodule development. With jointvetch new lateral roots naturally develop from mature regions of the tap root, and these lateral roots are capable of nodulation. Autoregulation, the partial restriction of nodulation in regions of the tap root that develop after inoculation, was evident in both hosts.

Keywords: Autoregulation, *Rhizobium*, lateral roots, tap root, wound entry

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1. Introduction

In many legumes the mode of bacterial entry into the host is through infection threads, which develop almost exclusively in immature root hair cells. Because mature root hairs are generally resistant to infection, the region of the tap root that contains immature and emerging root hairs at the time of inoculation is the most susceptible to nodule initiation (Bhuvaneshwari et al., 1980, 1981, 1988; Eskew et al., 1993). In root tissue that develops after inoculation, and subsequent to the initiation of adequate nodules, excessive nodulation is suppressed by a mechanism referred to as feedback inhibition or autoregulation (Pierce and Bauer, 1983; Caetano-Anollés and Gresshoff, 1991a). Genetic analysis has demonstrated that autoregulation is conditioned by a few genes, and that the host shoot is intimately involved in producing the signal that inhibits excessive nodulation (Caetano-Anollés and Gresshoff, 1990, 1991b; Gerahty et al., 1992; Gremaud and Harper, 1989).

Members of several tropical legume genera, including *Aeschynomene* (Arora, 1954), *Arachis* (Chandler, 1978), *Neptunia* (James et al., 1992), *Sesbania* (Ndoye et al., 1994), and *Stylosanthes* (Chandler et al., 1982), produce root nodules at the juncture of lateral root emergence. Although root hairs are abundant at the base of the lateral roots of some of these hosts, and, at least in peanuts, are generally absent elsewhere, infection is not accomplished through root hairs. Instead, the rhizobia grow between cortical cells at the base of the lateral root and invade some of the host cells, which then begin to divide, to form the infected core of the nodule. Because emergence of lateral roots causes a crack in the tap root cortex, this mode of infection has been described as "crack, or wound, infection" (Sprent and Sprent, 1990). However, in the cases where infection was carefully examined, bacteria actually infect cells of the lateral root cortex, not the tap root cortex (Chandler, 1978; Ndoye et al., 1994; Chandler et al., 1982). Thus, the precise importance of a "wound" in the tap root cortex remains undefined. The exception to a lateral root origin of the nodular tissue may be *Aeschynomene*, where Arora reported that the nodules arose from the tap root pericycle (Arora, 1954). However, while Allen and Allen (1940) had originally described the origin of *Arachis* nodules to be pericyclic, it was determined by Chandler (1978) that they actually originate from lateral root cortical cells. A reappraisal of *Aeschynomene* might determine that it also follows the common pattern.

In this study we have characterized the developmental kinetics of nodulation in *Arachis hypogea* (peanuts) and *Aeschynomene americana* (jointvetch). Lateral root maturity was identified as a key determinant of susceptibility to nodulation, but no relationship with tap root maturity was

found. As in other legumes, autoregulation limits nodulation in distal portions of the developing root system.

2. Materials and Methods

Plant growth and inoculation

Seeds of peanut (*Arachis hypogea* L.) cv. Spanish, were obtained from W.A. Burpee Co. (Warminster, PA) and seeds of jointvetch (*Aeschynomene americana* L.) were provided by Dr. K. Quisenberry (University of Florida, Gainesville). Rhizobial strains *Bradyrhizobium* spp. USDA3456 and USDA3516, were provided by Peter van Berkum (USDA National *Rhizobium* Collection, Beltsville, MA), and used to inoculate peanut and jointvetch, respectively, after culturing in YEM (Vincent, 1970). Prior to planting, seeds of peanut were immersed for 5 min in 95% ethanol, and seeds of jointvetch were surface sterilized for 5 min in a 1:1 dilution of household bleach, followed by thorough rinsing in tap water. Seeds were germinated in vermiculite for 3 days, at 28°C in the dark, until roots had grown approximately 4 cm. The seedlings were then transferred to minirhizotrons, constructed of two glass plates, (35 × 55 cm each) separated by a strip of Styrofoam, 2.7 cm thick, placed along both sides and across the bottom of the assembly. The plates and spacers were secured with duct tape. Prior to assembly, one of the glass plates was lined with seed germination paper, removed from Seed Pack Growth Pouches (Vaughan's Seed Co., Downers Grove, IL). The space between the glass plates was then filled with medium grade vermiculite (Waldo & Assoc., Perrysburg, OH), watered with 1× Jensen's nitrogen free nutrient solution (Vincent, 1970), and seedlings placed between the germination paper and the glass plate. Either 2 or 4 seedlings were placed in each assembly, for peanuts or jointvetch, respectively. The assembled minirhizotrons were placed at a 30° angle, in a growth chamber at 28°C, 16 hrs light. The outer surfaces of the assemblies were covered with aluminum foil to prevent exposure of the developing roots to light. Three additional days of growth in minirhizotrons produced rapid extension of the tap root, including development of numerous lateral roots. Plants were inoculated by pouring 100 mls of inoculum between the germination paper and the glass surface. Inoculum consisted of a 1:10 dilution of mid-log-phase bacterial cultures in 1× Jensen's nitrogen free nutrient solution. At the time of inoculation the ontological development of the root systems was recorded by simply removing the aluminum foil and photocopying the root systems, for peanuts, or tracing the root system onto a clear acetate sheet, for jointvetch, which has a much finer root system and is difficult to see on a

photocopy. The position of the root tip at the time of inoculation was recorded as the root tip mark, RTM, and provided a reference for later measurements relating root development to nodule distribution. Plants were watered as needed with tap water. Each experiment was replicated twice, with 14 or 28 seedlings per replicate, for peanuts and jointvetch, respectively. Because the results of each replication were essentially the same, the data was combined in the final analysis. Additionally, 4 to 8 uninoculated seedlings were included as controls in each replication.

Measurement of root growth and nodule appearance

Peanut root development was assessed at 3, 6, 12 and 24 days post inoculation by making photocopies of the root system. These permanent records were then scored for the appearance of additional lateral roots arising from the tap root, in 2 cm sections above and below the RTM. The appearance of additional lateral roots over time was noted with jointvetch by marking distances along the tap root on the glass surface, and counting the number of lateral roots within each 2 cm section above or below the RTM. Appearance of nodules was recorded on photocopies or transparency sheets, at 12 and 20 days for peanuts, and 8, 16, and 24 days for jointvetch.

To assess the relative importance of tap root versus lateral root maturation in peanut, five sterilized seeds were grown for 16 days in vermiculite, uprooted, trimmed to a tap root length of 8 cm, stripped of all lateral roots by pulling them out of the tap root, and then replanted in fresh vermiculite. The plants were inoculated with rhizobia at the time of replanting. After an additional three weeks of growth the plants were uprooted again and scored for nodulation on the replacement lateral roots.

The importance of a lateral root meristem in determining the susceptibility of lateral root bases to nodulation, in peanuts, was examined by decapitating 251 lateral roots that were at a susceptible age for nodulation. Specifically, five minirhizotrons planted with peanuts were grown and inoculated as above. Two days after inoculation of 6 day-old seedlings the glass plate in contact with the roots was removed, and each root system was treated by removing all of the lateral roots, of 2.5 cm or less in length, present on the right side of the tap root, leaving approximately 3 mm of lateral root base. The glass plate was replaced, and the assembly returned to the growth chamber for 14 additional days before scoring each lateral root for nodulation. The undisturbed lateral roots on the left side of each plant, within the same age range, were also counted for nodulation, as controls.

3. Results

Peanut root development was very geometric; lateral roots developed from the large tap root in a moving wave, much as root hairs emerge on many legumes. When these roots were scored and then compiled the pattern appears as distinct blocks of roots appearing over time (Fig. 1A). On average, approximately 8 to 10 lateral roots emerge from each 2 cm section of tap root, although the number declined as the tap root had reached a total length of 30 cm or more. An important aspect of lateral root development in peanuts is that once a developing segment of tap root produced lateral roots, that section of tap root did not develop additional laterals during the course of our experiments, i.e., from day 6 to day 20 virtually no new laterals appeared.

Nodule development on the tap root of peanuts was most abundant in a region centered around 8 cm above the RTM (Fig. 1B). At the time of inoculation the average distance between the RTM and the youngest emergent lateral root was 7.0 ± 0.45 cm (SD), indicating that the most susceptible zone was that region in which lateral roots were just developing. Additional nodules that appeared between days 12 and 20 primarily reflect the addition of second and third nodules at the base of lateral roots that had already formed nodules, rather than the late appearance of nodules on mature laterals. To confirm that the lack of nodulation below the RTM was not due to a physiological restriction of root development near the bottom of the minirhizotrons, 10 plants were grown until the RTM was below 30 cm and then inoculated. The young and emerging lateral roots, all at the bottom of these minirhizotrons, developed nodules at a frequency of 27% (81/298), similar to lateral roots near the top of other minirhizotrons. Additionally, uninoculated control plants did not develop nodules in any experiment.

The restriction of nodulation in the tap root region having well developed lateral roots at the time of inoculation could reflect either maturation of the tap root or of the lateral roots. When lateral root length at the time of inoculation was compared to frequency of nodulation (Fig. 2), it was found that the shortest roots were most likely to develop nodules, and that lateral roots over 2 cm in length were not likely to develop nodules. These results suggested that lateral root maturity was important in delimiting the developmental window for nodulation. However, because lateral root development in peanuts is very synchronous, lateral root length would be highly correlated with tap root maturity. To test the role of tap root maturity, nodulation of emergent lateral roots on an older tap root was measured. Five plants that had been stripped of all lateral roots and then replanted and inoculated grew a new set of laterals after several days, all originating from the original tap root tissue, as no further growth of the tap root occurred. After 3 weeks these tap roots

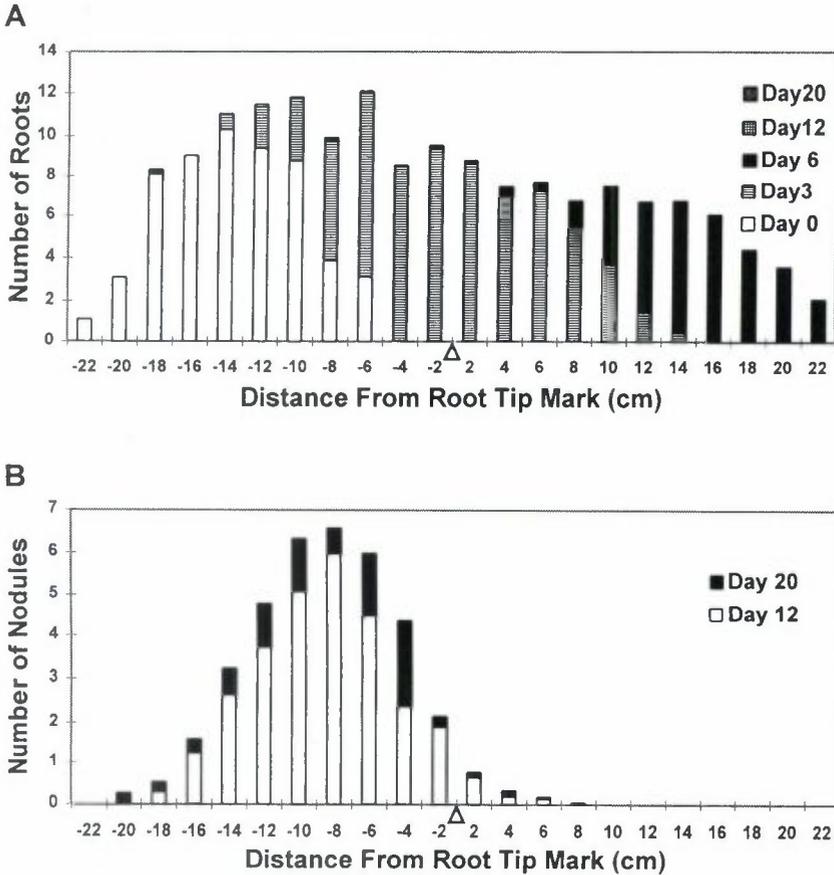


Figure 1. Kinetics of cumulative lateral root emergence (A), and nodulation (B), on peanut tap roots. Seedlings, grown in minirhizotrons, were six days old at the time of inoculation, depicted as day 0. The root tip at the time of inoculation is marked by a triangle on the X axis; each column represents the average number of roots or nodules appearing within a 2 cm segment of tap root; positive numbers indicate tissue that developed subsequent to inoculation, while negative numbers depict distance above the root tip. The number of new roots appearing at days 12 and 20 were too few to appear on the graph. The average of two independent experiments is given, with 14 plants in each replication.

averaged 31.6 ± 2.8 (SD) nodules on the replacement laterals, demonstrating that tap root maturity did not preclude lateral root nodulation. When lateral root meristems were removed from lateral roots two days after inoculation there was very little effect upon nodule development. Totaled over ten plants,

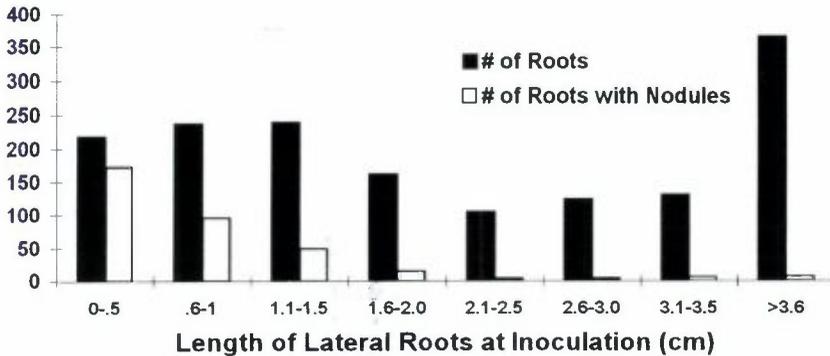


Figure 2. Frequency of nodule formation on peanut lateral roots of different lengths at the time of inoculation. At the time of inoculation, 6 days after seed imbibition, the length of each of the lateral roots on 28 plants was determined and recorded. Twenty days after inoculation these same lateral roots were scored for the presence of at least one nodule at their base.

25% (63/251) of the lateral roots that had been severed developed nodules, while 30% (77/249) of the undisturbed lateral roots nodulated.

In the case of jointvetch growth of the tap root was followed by the emergence of lateral roots, but unlike peanut, additional lateral roots continued to appear at later times (Fig. 3A). This is most evident at 16 days after inoculation, when a large number of new lateral roots were scored in the upper region of the tap root. Eight days after inoculation, nodules were most abundant about 6 cm above the RTM (Fig. 3B). Because lateral roots of jointvetch only rarely developed more than one nodule, the appearance of additional nodules over time reflects the nodulation of additional lateral roots. Specifically, if only those nodules that appeared between day 16 and 24 are considered, there is a very broad distribution, reflecting the fact that new lateral roots are continuing to emerge along the length of the tap root, and some of these are then developing nodules. The initial appearance of nodules in a tight cluster indicates that jointvetch, like peanut, restricts nodulation, through lateral root maturity and autoregulation, within a developmental window. When the length of lateral roots at the time of inoculation is compared to frequency of nodulation (Fig. 4), it is evident that lateral roots greater than 2 cm in length are resistant to nodulation. That this restriction is defined by lateral root maturity and not tap root age is evident by the continued appearance of newly nodulated young lateral roots over the entire area of the tap root.

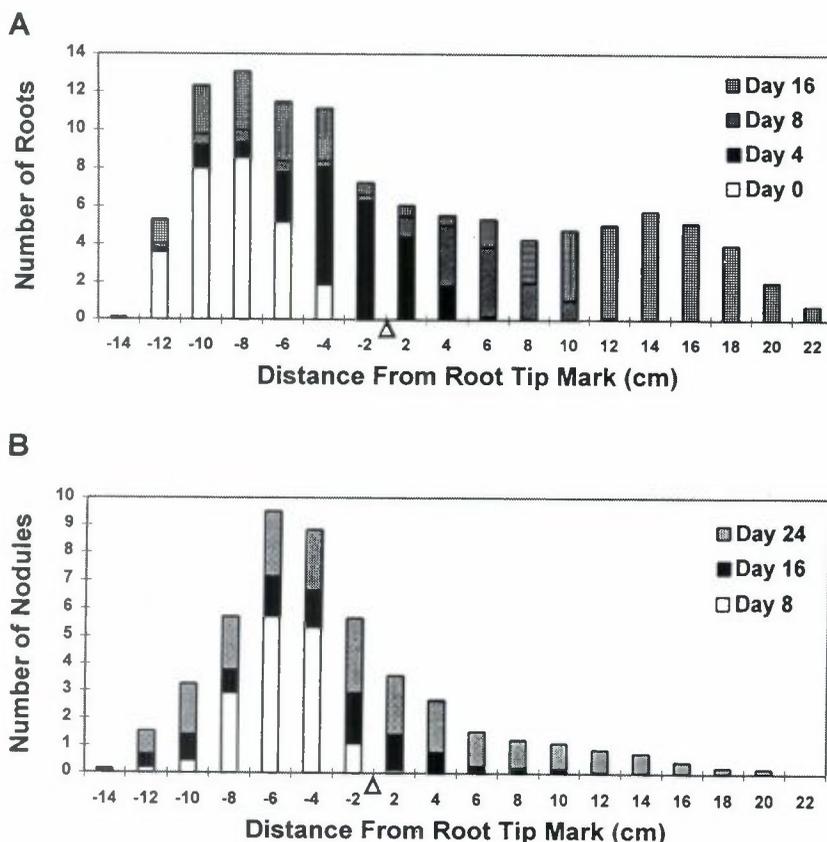


Figure 3. Kinetics of cumulative lateral root emergence (A), and nodulation (B), on jointvetch tap roots. Seedlings, grown in minirhizotrons, were six days old at the time of inoculation, depicted as day 0. The root tip at the time of inoculation is marked by a triangle on the X axis; each column represents the average number of roots or nodules appearing within a 2 cm segment of tap root; positive numbers indicate tissue that developed subsequent to inoculation, while negative numbers depict distance above the root tip. The average of two independent experiments is given, with 28 plants in each replication.

4. Discussion

To date, the developmental regulation and kinetics of nodulation has been studied only in legumes that develop nodules following bacterial invasion of susceptible root hairs. In these legumes it is clearly the maturity of the root hairs that restricts the development of nodules on more mature regions of the

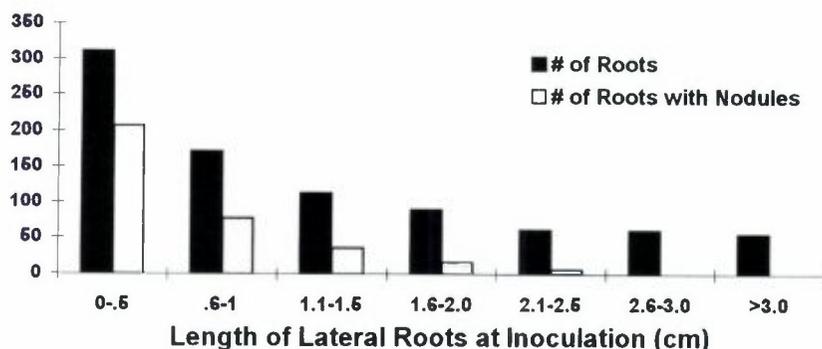


Figure 4. Frequency of nodule formation on jointvetch lateral roots of different lengths at the time of inoculation. At the time of inoculation, 7 days after seed imbibition, the length of each of the lateral roots on 28 plants was determined and recorded. Twenty-four days after inoculation these same lateral roots were scored for the presence of at least one nodule at their base.

root (Bhuvanewari et al., 1980, 1981, 1988, Eskew et al., 1993). Bhuvanewari et al. (1981) used growth pouches to study the transient susceptibility to nodulation in four legumes, including peanut. For three of these legumes; cowpea, alfalfa, and white clover, root hair maturity at the time of inoculation was a reliable indicator of nodulation competence. Although some nodulation occurred in the mature root hair zone of white clover this may reflect the presence of a few immature root hairs in this region. In hydroponically grown soybean, root hairs on mature regions of the root are susceptible to infection (Selker et al., 1988). Thus, while an immature root hair appears to be required for nodulation in most cases, a mature tap root does not preclude nodule development.

The fourth legume examined by Bhuvanewari et al. (1981) was peanut. However, due to the large size of peanut seeds and tap roots, pouches were not a suitable growth container. As a result, their observations of this host were preliminary, although they did suggest that root hair maturation was correlated with a loss of susceptibility to nodulation. To overcome the limitations of growth pouches in the study of peanut nodulation we have constructed minirhizotrons, and used them to examine nodulation kinetics in two legumes which nodulate at the site of lateral root emergence. These minirhizotrons were especially useful for jointvetch because we were able to map the entire root system, rather than the 13 cm that would have developed

in a growth pouch. The distribution of jointvetch nodules, 24 days after inoculation, covered a full 30 cm.

Our results indicate that in each host lateral root maturity is the primary factor in determining nodulation competence. The maturity of the tap root itself is not important. These results are consistent with the observation that, in peanut, the bacteria actually infect the cortical cells of the lateral root, and not the cortical cells of the tap root (Chandler, 1978). Although root hairs are not directly involved in nodulation of peanut (Allen and Allen, 1940; Chandler, 1978) we examined root hair development in relation to lateral root length and nodulation. Root hairs arose in tufts at the base of all lateral roots, and continued to grow in length for several days, a period in which the lateral roots grew to ≥ 2 cm in length. There was considerable variation in the length of root hairs when they matured, which made it very difficult to determine when the root hairs on a given lateral root had become "mature". Thus it was difficult to correlate root hair maturity with nodulation competence; we found that lateral root length was a more reliable predictor.

Because hormonal balances are implicated in several stages of nodulation (Hirsch and Fang, 1994) we considered the hypothesis that the inhibition of nodulation on older lateral roots might reflect a changing hormonal gradient due to the increasing distance between the lateral root's apical meristem and the basal cells. By removing the apical meristems of young lateral roots we have shown that their presence is not required for nodule development. Because the lateral roots were not removed until two days after inoculation, this experiment does not address control of nodule initiation in maturing lateral roots.

In the distal portions of peanut tap roots autoregulation clearly restricted nodule development. In jointvetch, an initial clustering of nodules just above the RTM suggests that an autoregulatory response is present, but the continued production of lateral roots, and nodules, over the length of the tap root begins to broaden this clustering after an additional 24 days. The autoregulatory response in soybeans has been shown to inhibit the transition of early cell division foci into truly meristematic stages (Gerahty et al., 1992). This aspect remains to be addressed in peanuts and jointvetch.

Finally, we have detected an interesting difference in the pattern of lateral root emergence in two legumes that normally develop deep tap root systems in sandy, xeric soils. In our minirhizotrons peanut tap roots developed lateral roots in an orderly progression, much as a pine tree develops branches. Over the next three weeks very few lateral roots (<1 per plant, on average) emerged from the tap root once this flush was complete. In jointvetch, lateral root emergence was not synchronized, with short and long laterals interspersed, and

new laterals routinely appeared from older tap root tissue, for at least the following three weeks.

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