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Light Microscopy Study of Nodule Initiation in *Pisum sativum* L. cv Sparkle and in Its Low-Nodulating Mutant E2 (*sym* 5)[1]

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ABSTRACT

We compared nodule initiation in lateral roots of *Pisum sativum* (L.) cv Sparkle and in a low-nodulating mutant E2 (*sym* 5). In Sparkle, about 25% of the infections terminated in the epidermis, a similar number stopped in the cortex, and 50% resulted in the formation of a nodule meristem or an emerged nodule. The mutant E2 (*sym* 5) was infected as often as was the parent, and it formed a normal infection thread. In the mutant, cell divisions rarely occurred in advance of the infection thread, and few nodule primordia were produced. Growing the mutant at a low root temperature or adding Ag^+ to the substrate increased the number of cell divisions and nodule primordia. We conclude that, in the E2 line, the infection process is arrested in the cortex, at the stage of initial cell divisions before the establishment of a nodule primordium.

Pisum sativum (L.) cv Sparkle forms many small nodules on its lateral roots, mostly within 2.5 cm of the primary root. Sparkle was mutagenized to induce symbiosis (*sym*) gene mutants defective in nodulation (8). Seven mutants at the *sym* 5 locus were independently selected as having few or no nodules (7, 8). The *sym* 5 mutants are temperature sensitive; in growth rooms, a few nodules consistently form at a cool root temperature (12°C), whereas nodules rarely develop at higher root temperatures (20–23°C) (3). At both temperatures, nodulation of the *sym* 5 mutants is enhanced if roots are treated with aminoethoxyvinylglycine or Ag^+, which are inhibitors of ethylene formation or action, respectively (4). This suggests that, in the *sym* 5 mutants, some stage of nodule formation is oversensitive to ethylene.

Nodule development in peas has been described by a number of authors (10, 14). Briefly, after root hair curling and infection thread formation, the infection thread branches in the outer cortex and grows toward the inner cortex. In the inner cortex, anticlinal cell divisions occur in advance of the thread and a nodule primordium is established. The infection thread branches grow into this center of dividing cells, releasing bacteria from the tips of the threads. A nodule meristem forms at one end of the nodule primordium, and eventually an infected nodule emerges from the root.

In this study, we compared a mutant *sym* 5 line with its near-isogenic nodulating parent to determine (a) whether the initial infection is normal, (b) at what stage nodule formation is blocked, and (c) how the *sym* 5 phenotype is altered by cool temperature or by addition of Ag^+.

Because it was impractical to study the entire root, we conducted a census of the infection stages in two representative portions of lateral roots.

MATERIALS AND METHODS

Seeds of *Pisum sativum* (L.) cv Sparkle and of the mutant E2 (*sym* 5) were surface sterilized for 8 min with 5% (v/v) aqueous household bleach and rinsed several times with sterile distilled water. Seeds were planted individually in 3.5- × 20-cm conical pots (Ray Leach Conetainer Nursery, Canby, OR) filled with coarse vermiculite wetted with nutrient solution (3). The plants were subirrigated with nutrient solution and grown under high pressure sodium vapor and metal halide lamps (550 μmol m^{-2} s^{-1} PPFD) with a day/night cycle of 16/8 h. Roots were kept at a constant temperature of either 12 or 23°C, and the shoots were at 20/15°C day/night temperatures.

Seedlings were inoculated 7 DAP[2] with 5 mL of a 1:100 dilution of *Rhizobium leguminosarum* bv *viceae* 128C53 (Nitragin Co., Milwaukee, WI) grown in yeast-mannitol broth.

Some pots at 23°C were treated at 5 and 12 DAP with 20 mL of 10^{-5} M Ag_2SO_4 in nutrient solution. Plants were harvested 19 DAP, visible nodules counted, and segments of the roots taken for microscopic examination. Some inoculated plants, growing at a constant root temperature of 23°C, were harvested 28 DAP.

For microscopic examination, the third and 14th lateral roots, counted from the cotyledons down, were harvested from 24 plants each of Sparkle and E2, for each growth condition. Transverse hand sections of these two laterals were made to obtain the segment from 1 to 1.5 cm distal to the site of branching from the primary root. These segments were sectioned longitudinally, stained with 0.05% toluidine blue O for 2 min, rinsed in distilled water, and observed with bright-field optics. For autofluorescence study, some sections were kept unstained and observed with epifluorescence optics.

To score the stages of nodule development, each longitudinal section was scanned on both sides, from the top of the

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[2] Abbreviation: DAP: days after planting.

root hairs to the middle of the stele. The stages of nodule formation, depicted in Figures 1 to 8, were defined as follows: stage a, infection thread arrested in root hair (Fig. 1) or in the basal epidermal cell bearing the infected root hair (Fig. 2); stage b, infection thread in outer or inner cortex with no cell division visible (Figs. 3–5); stage c, infection thread branches associated with cell divisions in the inner cortex (Fig. 6); stage d, nodule primordium (small organized group of cells in the earliest stage of differentiation) (Fig. 7); stage e, emerging nodule with characteristic round shape (Fig. 8); stage f, emerged nodule.

The number of infections at each stage was recorded, and the total number of infections was calculated per centimeter of lateral (secondary) root. The total number of initiated tertiary roots (primordium, emerging, or emerged) was also noted.

In the plants grown for 28 d, we recorded the number of visible emerged nodules in the same two root zones as studied for microscopy.

RESULTS AND DISCUSSION

Nodulation of Entire Root

Most nodules were on lateral roots, usually within 2.5 cm of the primary root. Sparkle, on average, had 140 nodules per plant when grown at a constant root temperature of 23°C (Table I). When Ag^+ was added to the substrate, nodule numbers on lateral roots of Sparkle were approximately doubled (Table I). At a root temperature of 12°C, the shoots of parent and mutant were shorter and had less dry weight (data not shown). There was also less growth of the root and fewer nodules (Table I). In all conditions tested, E2 had fewer nodules than Sparkle (Table I). Both low temperature and Ag^+ increased nodulation of the mutant. These results are similar to those reported previously (3, 4).

Nodules on primary roots were rare (Table I), and when present, they were larger than those on laterals. Few were found on Sparkle in either of the three treatments, and none was visible on E2. Cool root temperature (12°C) significantly increased the number of nodules on Sparkle primary roots compared to 23°C.

Nodule Development in E2 (*sym* 5)

The infection thread in the mutant was normal (Figs. 1–8). It was similar to that of Sparkle (not shown) and had the typical features described in the literature. In both lines, many infections were arrested early in the process, either in the root hair (Fig. 1) or in the basal epidermal cell bearing the infected hair (Fig. 2). Infection threads stopped at this stage had a tuberculated surface or bore many short branches (Figs. 1 and 2). Nutman (16) observed these features in aborted threads of several species.

At the point of passage between cortical cells, the infection thread in E2 had the normal funnel shape (Fig. 4) described by Thornton (20) in alfalfa roots. In E2, as in Sparkle, the infection threads were often adjacent to large nuclei (Fig. 4) as noted by Nutman (16) and Newcomb *et al.* (15). The threads were autofluorescent. The younger portions of the infection thread did not stain with toluidine blue O (Fig. 5) showing that they were histochemically different from the older parts of the thread. This result was similar to observations made by McCoy (13) on alfalfa and by Newcomb (14) on pea.

The infection threads usually branched when they reached the outer layers of cortical cells (Figs. 3, 5, and 6). In both lines of cv Sparkle, specific foci of cell division were rarely observed ahead of the infection thread at this stage. (This differs from the observation in cv Little Marvel that cortical cell divisions could occur while the thread was still in the root hair [15].) As we will tabulate later, most infections in the mutant were blocked at the next stage, *i.e.* the branched infection thread penetrated into the inner cortex, but there were no cortical cell divisions in advance of it (Fig. 3).

When the mutant was induced to nodulate, nodule development was normal (Figs. 6–8) and indistinguishable from that in Sparkle (not shown). Thread branches entered divided cells (Fig. 6) and penetrated the nodule primordium (Fig. 7), and a nodule formed with a distinct meristematic zone (Fig. 8).

Number of Infections and Number of Tertiary Laterals

In the two portions of lateral roots examined, there were no significant differences between Sparkle and E2 in the total number of infections at 23 or 12°C or in the presence of Ag^+ (Table II). The standard errors of the means were large: this is because at 23 and 12°C some plants in both lines did not have any infections, whereas some had as many as six to 10 in the 0.5-cm segments examined. The number of infections in the Ag^+-treated plants was even more variable. Four samples of lateral 3 and eight samples of lateral 14 for each line did not have any infections, whereas the highest number of infections was 18 in a segment.

Under each growth condition tested, the mutant did not differ from the parent in the frequency of tertiary roots forming on the laterals (Table II). This extends our previous observations that, except for defective nodulation, the mutant line E2 does not differ morphologically from normal (3).

Stages of Infection in Sparkle and E2

Figures 9 to 11 indicate the stages of nodule formation observed in the sampled segments of two lateral roots. In general, the number of emerged nodules on these segments correlates well with the total number of nodules on the roots (Table I).

23°C Treatment

At 23°C, in both laterals of Sparkle (Fig. 9, A and B), approximately one-quarter of the infections were arrested in the epidermis (column a). A slightly smaller number of threads had stopped within the cortex (column b), and threads associated with cortical cell divisions were rare (column c). About half of the infections resulted in a nodule meristem (columns d and e) or an emerged nodule (column f).

When Sparkle plants were grown for 28 d, the visible emerged nodules on the two root segments were larger but not more numerous than at 19 d. The number of emerged

Table I. *Effect of root temperature and of Ag^+ on Nodulation of Sparkle and Its Mutant E2 (sym 5)*

Each value is the mean ±SE of 24 plants at 19 DAP. All counts of nodules for the lateral roots are significantly different from each other (*t* test, $P \leq 0.05$).

Root Environment	Root Type	Total Nodules/Plant	
		Sparkle	E2 (*sym* 5)
23°C	Lateral	138 ± 14	0.12 ± 0.09
	Primary	8 ± 2[a]	0
12°C	Lateral	95 ± 8	7.5 ± 1.1
	Primary	16 ± 2	0
23°C + Ag^+	Lateral	292 ± 16	21 ± 2
	Primary	11 ± 2[a]	0

[a] The only counts of nodules for primary roots not significantly different at a 5% confidence level.

nodules was 2.33 ± 0.87 and 1.67 ± 0.44/cm on laterals 3 and 14, respectively, compared to 1.83 ± 0.42/cm on plants at 19 d (Fig. 9, A and B, column f). Thus, the infections measured within the root at 19 DAP were truly blocked and not merely slower to emerge.

In E2, a large proportion of threads were blocked in the epidermis (Fig. 9, C and D, column a) and in the cortex (column b). The major difference between mutant and parent was that, in E2, few infection threads in the cortex were associated with cell divisions (column c), only a few primordia occurred (column d), and no nodules formed (columns e and f). In both laterals of the mutant, about 55% of the infections were arrested in the cortex, *i.e.* before the establishment of a nodule primordium.

12°C Treatment

At 12°C, in Sparkle, the distribution of infection stages in the two root segments differed only slightly (Fig. 10, A and B) from the pattern observed at 23°C (Fig. 9, A and B). Fewer infections aborted in the epidermis (column a). Approximately 65% of the infections succeeded in forming either a nodule primordium (column d), an emerging nodule (column e), or an emerged nodule (column f). There is an apparent discrepancy with the total number of nodules per plant (Table I). This inconsistency can be explained by the fact that, at 12°C, most nodules were found on the oldest laterals (1–20). There were almost none on the younger roots, whereas at 23°C nodules were found on laterals throughout the root system.

In E2, lateral 3 had fewer infection threads arrested in the epidermis than at 23°C and lateral 14 had more (Fig. 10, C and D, column a). As at 23°C, most of the infections in the mutant were arrested in the cortex but in a smaller percentage (38 *versus* 55%). At 12°C, more threads in the inner cortex were found associated with cell divisions (column c). This indicates that the cool temperature permits formation of nodule primordia (column d), emerging nodules (column e), and mature nodules (column f). When nodules do form on the mutant, their development is normal (Figs. 6–8).

Ag^+ Treatment

Treatment of roots with Ag^+ increased the number of emerged nodules on the two portions of lateral roots of Sparkle (Fig. 11, A and B) compared with the untreated plants at 23°C (Fig. 9, A and B). This agrees with the increased total nodulation on the plant (Table I). The treatment seems to act on early steps of the infection process; fewer infections were blocked in the epidermis (column a) and in the cortex (column b).

In the mutant, Ag^+ promoted nodulation (Fig. 11, C and D; Table I), apparently by decreasing the number of infections stopped in the cortex (column b) and by increasing the number of cell division centers associated with the infection threads (column c). Such cell divisions were seldom observed on the untreated samples (Fig. 9, C and D). There were nodule primordia as well as emerging and emerged nodules on the lateral roots treated with Ag^+ (columns d–f), whereas none was observed on the untreated roots.

We studied 0.5-cm segments of two separate lateral roots because lateral roots may not behave similarly throughout the root system. The first lateral roots to emerge are initiated in the embryo before germination (5), and it is possible that their

Figure 1. Infection thread (arrows) in a root hair on a lateral root of E2. The thread is growing toward the base of the hair (bottom right). Its tip is expanding into large protuberances (*). The thread has a tuberculated and rough surface. Bar, 45 μm.

Figure 2. This infection thread (arrows) in an epidermal cell (EC) of E2 is probably abortive. It has protuberances and does not grow toward the cortex (C) but rather remains in the epidermis. Bar, 95 μm.

Figure 3. An infection thread (arrows) in a lateral root of E2. It is well developed and branched in the inner cortex, but no cell divisions are associated with its growth. Bar, 230 μm.

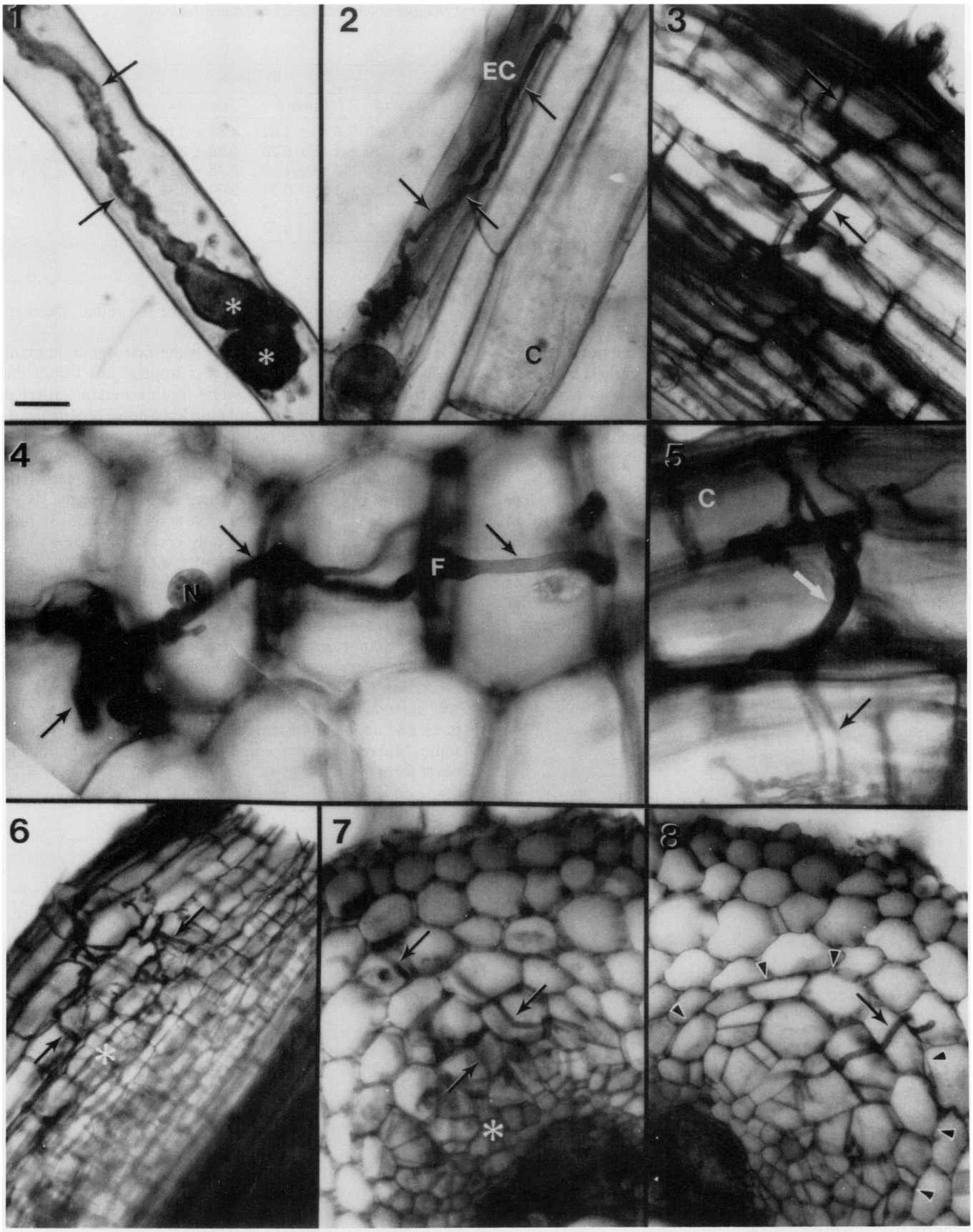
Figure 4. An infection thread (arrows) in the cortex of a lateral root of E2 enlarges when entering a new cell, forming a funnel-shaped structure (F). A large nucleus (N), in prophase, is close to the thread. Bar, 80 μm.

Figure 5. An infection thread (white arrows) is passing through the outer cortex of a lateral root of E2. It branches when reaching the first layer of cortical cells (C). Newly formed branches (black arrows) are histochemically different from older ones; they do not stain pink with toluidine blue O. Bar, 95 μm.

Figure 6. The infection thread (arrows) in this E2 lateral root has numerous branches reaching a center of divisions (*) in the inner cortex of the root. Bar, 230 μm.

Figure 7. When reaching the inner cortex of the root, the branches (arrows) of the infection thread enter a nodule primordium (*) composed of cells that have been induced to divide earlier in the development of the nodule. The nodule does not yet have a determinate shape. Bar, 230 μm.

Figure 8. Emerging nodule (arrowheads) in an E2 lateral root. The infection thread (arrow) is still visible in the newly developed organ. Bar, 230 μm.



Figures 1–8.

Table II. *Effect of Root Temperature and of Ag^+ on the Number of Infections and of Tertiary Roots in Two Segments of Lateral Roots of Sparkle and E2*

Each value is the mean ± SE of 24 plants at 19 DAP

Root Environment	Parameter measured	Sparkle		E2 (*sym* 5)	
		Lateral 3	Lateral 14	Lateral 3	Lateral 14
23° C	Total infections/cm	5.66 ± 0.74	4.42 ± 0.80	6.74 ± 1.02	4.66 ± 0.74
	Tertiary roots/cm	0.92 ± 0.22	0.74 ± 0.20	1.16 ± 0.26	0.58 ± 0.22
12°C	Total infections/cm	7.58 ± 0.90	6.00 ± 0.86	7.16 ± 1.10	5.76 ± 0.74
	Tertiary roots/cm	1.82 ± 0.38	1.24 ± 0.38	1.58 ± 0.34	2.00 ± 0.48
23°C Ag^+	Total infections/cm	5.92 ± 0.96	5.84 ± 1.38	7.26 ± 1.68	7.50 ± 1.30
	Tertiary roots/cm	1.50 ± 0.32	1.32 ± 0.32	1.50 ± 0.32	1.32 ± 0.26

development differs from those roots initiated after the tap root has emerged. If nutrients or growth regulators from the cotyledons are involved in controlling nodule number (18), then younger laterals might differ from the earliest laterals to form. In our experiments, however, there was no significant difference between the two laterals we sampled. For each line, there were generally fewer infections on lateral 14 than on lateral 3 (Table II); this was expected because lateral 14 had not yet emerged when the plants were inoculated 7 DAP.

Nodule numbers are commonly extremely variable on replicate plants. Similarly, we found large variability in infection numbers within two representative portions of lateral root. The large sample size of our study (24 plants/treatment), however, permits some confidence in the observations. For each treatment, the total number of infections did not differ between Sparkle and its mutant (Table II). Thus, the defect controlled by *sym* 5 does not involve the initial stages of infection.

To our knowledge, E2 (*sym* 5) is the first legume mutant in which the nodulation process is stopped just before the inner cortical cells divide. Several legume mutants selected for abnormal (no or low) nodulation have now been obtained. None of these have their nodulation stopped at a stage similar to that of E2; most are blocked earlier. For example, the alfalfa mutant MnNC-1008 (NN) is defective in root hair curling and cell divisions (2). Three nonnodulating soybean mutants are blocked at early stages: nod49 and nod139 lack curled root hairs, and nod772 only occasionally shows hair curling (11). The chickpea mutant PM233B does not form infection threads (12). In nonnodulating pea mutants, Postma *et al.* (19) found that mutant K24 had curled root hairs but did not form infection threads. On the other hand, pea mutant K5 formed more infection threads than its parent (unlike E2) but did not develop nodules. The stage at which infection was blocked in K5 was not reported. In the strain-specific pea cv Afghanistan, noninfective rhizobia promote deformed root hairs, and nodule meristems are initiated. However, although some "naked" bacteria enter root hairs, infection threads never form (9).

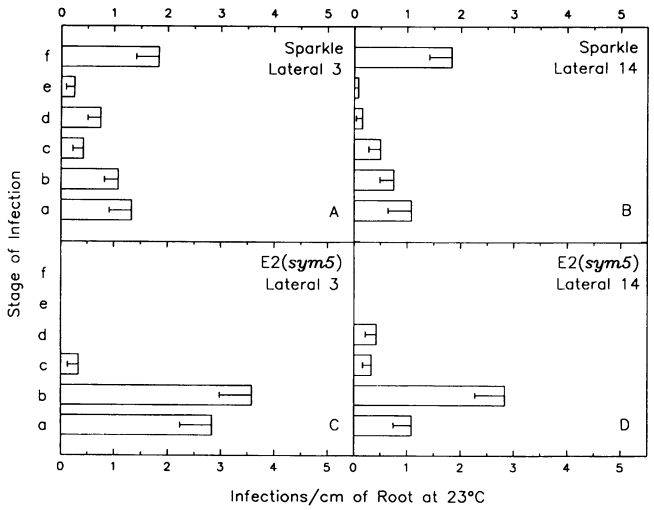


Figure 9. Number of infections at 19 d in two portions of lateral root growing at 23°C for Sparkle (A and B) and for E2 (C and D). The infections were classified as six different stages: a, infection thread arrested either in root hair or in epidermal cell; b, infection thread arrested in cortex; c, infection thread in cortex associated with cell divisions; d, nodule primordium; e, emerging nodule; and f, mature nodule. The total number of infections is in Table II. Each panel shows the results from 24 plants. Each column indicates the mean number (±SE) of infections at each stage.

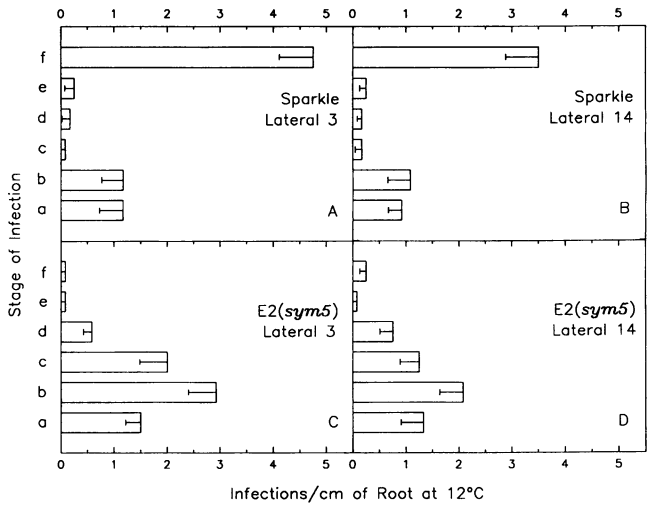


Figure 10. Number of infections in two portions of lateral root at 12°C. Description of symbols as in Figure 9.

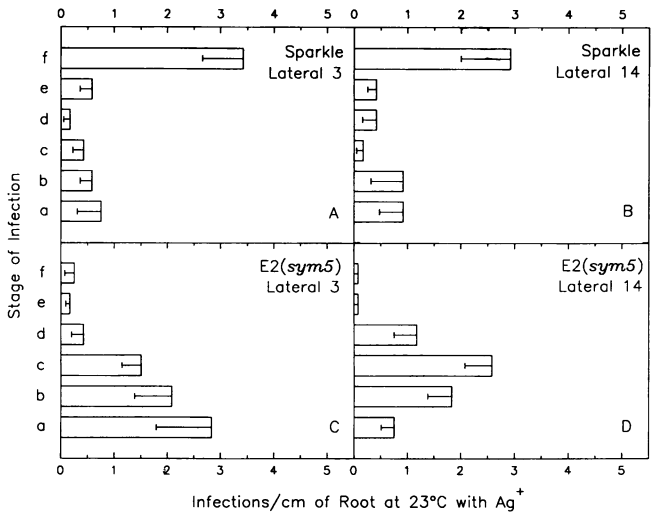


Figure 11. Number of infections in two portions of lateral root grown at 23°C and treated with Ag^+. Description of symbols as in Figure 9.

Ineffective *Rhizobium* strains have been studied, but again we were unable to find a report in which the nodulation process was blocked at a stage similar to that in E2. For example, *Rhizobium trifolii* region II mutants are capable of infecting *Trifolium subterraneum*, but the nodules are not as numerous as, and develop more slowly than when the plants are inoculated with, the wild-type *Rhizobium* (6). Cortical cell division is initiated by the mutants but is not as extensive as that induced by the parent strain. Several *R. trifolii* mutants studied by Pankhurst (17) form tumor-like growths and ineffective nodules on *Trifolium pratense* roots. Pankhurst (17) studied their structure and did not report blockage at the stage of cell division. *Rhizobium meliloti* mutated at *nod*A or *nod*C did not stimulate mitosis in the outer cortex of alfalfa (1).

Legumes regulate nodule numbers by aborting infections (10, 16). In Sparkle, and in the *sym* 5 mutant E2, approximately 25% of the infections are blocked in the epidermis. In addition, in the mutant, infection threads are seldom associated with cortical cell divisions. Nodulation of the mutant is enhanced by cool root temperature (which may lower ethylene production) and by Ag^+ (an inhibitor of ethylene action) (3, 4). However, we still lack direct evidence implicating ethylene in the inhibition of nodule primordium formation.

In the *sym* 5 mutant E2, the number of infections does not differ from that in parental Sparkle, and the infection thread appears normal. Nodule development, when it occurs, is normal. The unique phenotype of E2 is at an early stage of nodule primordium formation, when cortical cells seldom divide in advance of the infection thread.

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