Investigation of a non-nodulating cultivar of *Pisum sativum* [1,2,3]

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A non-nodulating cultivar of *Pisum sativum* cv. Afghanistan was studied to characterize the nature and location of the non-nodulating factor. Nodule formation was not temperature sensitive. *Rhizobium leguminosarum* could exist in the rhizosphere. Root secretions did not decrease nodulation in adjacent normal plants, nor did the proximity of normal plants promote nodulation. Infection threads formed in the root hairs, but nodules were not formed. The infection process apparently aborted, resulting in the formation of swellings on areas of the root where nodulation would normally occur. Grafting experiments indicate that the factor preventing nodulation is in the root and is not translocated from the cotyledon or plant top.

Symbiotic dinitrogen fixation in legumes involves a complex genetic and physiological interaction between host plant and infecting bacterium. Most researchers have concentrated on the microsymbiont, and the plant's contribution has received little attention. One approach to determining the role of the plant is the study of cultivars with a specific genetic effect on the processes of infection or fixation.

There are numerous reports in the literature where a host effect is inferred, but genetic analysis is usually lacking. Many such cases are described in Nutman's review (1). Holl and LaRue (2, 3) have tabulated the reports in which a specific effect in the legume has been reported and a host gene has been characterized. Five genes involved in the symbiosis have been reported in red clover (*Trifolium pratense* L.), four in soybean (*Glycine max* (L.) Merr.), and five in peas (*Pisum sativum* L.). Most of these genes are concerned with controlling nodule number or the effectiveness of the symbiosis. Four genes confer resistance to rhizobial infection or nodule formation.

In a non-nodulating cultivar of red clover, resistance to nodulation is conditioned by a simple recessive gene *r* (4). The physiological basis of the resistance is unknown. Lie (5, 6) described a temperature-sensitive cultivar of pea which does not nodulate if the roots are maintained at a low temperature. The single gene responsible has been designated Sym_1 (3). In soybean, the homozygote rj_1rj_1 is resistant to nodulation (7). The roots secrete a substance that inhibits nodulation even in adjacent normal soybean or clover plants (8), though the substance does not greatly decrease the number of *R. japonicum* in the rhizosphere (9).

Lie reported a pea cultivar from Afghanistan which is resistant to nodulation (6). The roots either do not nodulate or form only a few nodules late in the plant's growth (6). Holl has characterized the gene responsible, sym_2, as a simple recessive (10). This paper describes initial studies to determine the nature and location of the factors preventing nodulation in *Pisum sativum* cv. Afghanistan.

Materials and Methods

Seed of *Pisum sativum* cv. Afghanistan PRL H722 was obtained from Dr. F. B. Holl and was derived from seed originally obtained from Dr. T. A. Lie, Vakgroep Microbiologie, Landbouwhogeschool, Wageningen, The Netherlands. Seeds are small, brown, and wrinkled; flowers are purple; there are no unusual characteristics other than non-nodulation both in greenhouse and field. *Pisum sativum* cv. Trapper, a normal nodulating field pea, was obtained from local seed houses.

Scarification of seed before planting resulted in uniform germination. The seeds were treated with commercial pea inoculum (Hansen Inoculator Co., Milwaukee, Wisc.) at time of planting. Seed Pak Growth Pouches (CanLab Ltd., Edmonton, Alberta) were used for some experiments, but in most trials, seeds were planted in mason jars or plastic pots containing a 1:2 (v/v) mixture of quartz sand – horticultural grade vermiculite. For experiments requiring a sterile root system, plants were grown in the Leonard jar assembly as described by Vincent (11). The plant nutrient was a modification of

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[2]Taken in part from a thesis presented by T. L. Degenhardt to the Department of Soil Science, University of Saskatchewan, Saskatoon, Sask.

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Wilson's nitrogen-free medium (13): KH_2PO_4, 0.272 g; K_2SO_4, 0.349 g; $CaSO_4 \cdot 2H_2O$, 1.03 g; $MgSO_4 \cdot 7H_2O$, 0.247 g; H_3BO_3, 0.004 g; $MnCl_2 \cdot 4H_2O$, 0.99 mg; $ZnSO_4 \cdot 7H_2O$, 0.575 mg; $CuSO_4 \cdot 5H_2O$, 0.125 mg; $FeCl_3 \cdot 6H_2O$, 5.41 mg; $NaMoO_4 \cdot 2H_2O$, 0.103 mg; Na_2-EDTA$\cdot 2H_2O$,* 0.675 mg; H_2O, 1 litre.

Plants were grown in a growth chamber at 9000 – 10 000 lx with a light/dark regime of 16/8 h. The temperature was maintained at 18 °C.

For microscopic examination, roots were fixed 24 h in Carnoy's solution (ethanol – chloroform – acetic acid, 6:3:1), rinsed, and stored in 70% ethanol. Sections were rinsed with water and stained 5 s in 0.05% toluidine blue, pH 6.8 (14). Root sections for electron microscopy were fixed in 4% glutaraldehyde followed by osmic acid in buffer. The tissues were dehydrated with absolute ethanol at 0 °C, embedded in resin (15), and cut to a thickness of about 75 nm.

Cleft grafts (16) of stems or roots were made when the plants were 3–6 days old. Stem grafts were made above the cotyledon and below the first stipules. Root grafts were made below the cotyledon and above the first lateral. The grafted plant was kept moist in a humid atmosphere for 5 days. Grafted roots sent out laterals above the graft, and plants grafted above the cotyledon sent out shoots. These unwanted laterals and shoots were removed with a razor blade.

Cotyledons were removed from 2-day-old seedlings as soon as the epicotyl had emerged. The seedlings were watered once with Hoagland's nutrient (17) to provide nitrogen and thereafter with the nitrogen-free nutrient.

To estimate the number of rhizobia in the rhizosphere, roots of 2-week-old plants were blended 1 min in 100 ml nitrogen-free nutrient in a Waring blendor. Several 10-fold dilutions were made of the slurry and used as inoculants for surface-sterilized Trapper seeds growing in sterile Leonard jars (11). After 25 days, the seedlings were examined for nodulation and the number of rhizobia was estimated by the 'most probable number' method (12, 18).

Results and Discussion

Microscopic Examination

Root and root hairs stained blue to purple with toluidine blue. Infection threads stained red (14) and were visible in both cultivars. These observations indicate that lack of nodulation on Afghanistan is not due to any defect in this early stage of the infection process.

In Afghanistan plants 7–11 days old, swellings appeared on the roots which were indistinguishable from young nodules on Trapper. By 18–21 days, Trapper bore normal nodules while Afghanistan had white swellings on the roots. Examination of the plant cells in the swellings under the microscope showed them to be enlarged, devoid of distinguishing features, and with some plant cell walls broken. Nodule-type cells containing bacteroids were not observed.

The very rare nodules formed on Afghanistan were normal in appearance, except that the nodule cells contained more than the usual number of starch granules (2).

Root nodule initiation in peas begins with cell divisions in the inner cortex at some distance from the advancing infection thread (19). It appears that in Afghanistan, nodule formation is initiated but ceases before the bacteria invade the plant cells in the inner cortex. The plant cells enlarge and multiply, but the result is a root swelling rather than a nodule.

Number of Rhizobia in the Rhizosphere

The number of rhizobia on the roots of 2-week-old plants was 1.5×10^4 ($P = 0.024$) per root on Afghanistan and 4.6×10^5 ($P = 0.003$) per root on Trapper. Though Afghanistan had fewer rhizobia, it is unlikely that this is the cause of the non-nodulating character. The number observed should still be adequate since nodulation has been observed in legumes with as few as 89 rhizobia per root (20).

TABLE 1. Interaction of Trapper and Afghanistan grown together

Arrangement	Average number of nodules per plant ± SD*	
	cv. Afghanistan	cv. Trapper
AAA	0	—
TTT	—	62 ± 11
TAT	0	77 ± 19
ATA	0	45 ± 12

NOTE: Three plants of Trapper (T) or Afghanistan (A), or a combination, were grown in nitrogen-free nutrient in a Seed Pak pouch for 4 weeks, at which time their nodules were counted.
*SD, standard deviation.

TABLE 2. Effect of activated carbon on nodulation of Afghanistan and Trapper

Cultivar	Treatment	Average number of nodules per plant ± SD*
Trapper	—	159 ± 39
	+ Carbon	115 ± 45
Afghanistan	—	0
	+ Carbon	0

NOTE: Plants were grown in nitrogen-free nutrient in sand–vermiculite. For treated plants, the top 3 cm of potting material was activated carbon. The effect of carbon on nodulation of Trapper was not statistically significant.
*SD, standard deviation.

*EDTA, ethylenediaminetetraacetic acid.

TABLE 3. Effect of grafts on nodulation of roots of Afghanistan and Trapper

Graft	No. plants	Position of graft	Average no. nodules per plant ± SD*
Trapper	14	Ungrafted control	144 ± 59
Trapper/ Trapper†	6	Above cotyledon	72 ± 39
	2	Below cotyledon	47
Afghanistan	14	Ungrafted control	0
Afghanistan/ Afghanistan	10	Above cotyledon	0
	8	Below cotyledon	0
Trapper/ Afghanistan	10	Above cotyledon	0
	4	Below cotyledon	0
Afghanistan/ Trapper	10	Above cotyledon	91 ± 29
	3	Below cotyledon	93 ± 24

*SD, standard deviation.
†Scion/stock. The plants were grafted above or below the cotyledon when they were 3 to 5 days old.

Root Secretions Inhibiting Nodulation

If Afghanistan secretes a substance inhibitory to nodulation, as does a non-nodulating line of soybean (8), then we might expect an effect on nearby normal plants. When Afghanistan and Trapper plants were grown together in liquid nutrient in plastic pouches, there was no apparent effect (Table 1). In these clear pouches (15 × 15 cm), the roots of the three plants touched at many points. The nutrient volume, maintained at about 25 ml, was small, and compounds secreted by roots were not much diluted. Apparently Afghanistan roots do not secrete a compound inhibitory to nodulation, nor does Trapper secrete a compound which permits nodulation in Afghanistan. Similar results were obtained when plants were grown together in sand–vermiculite mixtures. Activated carbon decreased the number of nodules in Trapper (Table 2), an effect noted previously by Vantsis and Bond (21). That carbon did not promote nodulation on Afghanistan supported the hypothesis that the non-nodulating character is not due to an organic inhibitor secreted by the roots.

Effects of Plant Grafts

Table 3 summarizes results from experiments in which Trapper scion was grafted on Afghanistan stock, and vice versa. Ungrafted plants or grafts with the same cultivar served as controls. It is apparent that the control for nodulation or non-nodulation is in the roots, and no stimulatory factor is translocated from the plant stem, leaves, or cotyledon.

Grafting of the top of *Trifolium repens* (white clover) to a root of a poorly nodulating cultivar of *T. ambiguum* (Caucasian clover) resulted in a plant with large healthy nodules (22). However, results of our grafts are like those obtained with non-nodulating soybeans (23, 24), which indicate that the nodulation response was determined by the genotype of the host root.

There is contradictory evidence on the role of the cotyledon in nodule formation. Removal of both cotyledons reduces nodulation in soybeans (25) and alfalfa (26). In *Pisum sativum* cv. Alaska, removal of one cotyledon increases nodulation, an effect interpreted as being due to a cotyledonary inhibition of nodulation (27). Removal of cotyledons did not permit nodulation of Afghanistan (Table 4). It is likely that

TABLE 4. Effect of cotyledon removal on nodulation

Cultivar	Treatment	Nodules on plant ± SD*
Trapper	Control	88 ± 38
	Cotyledon removed	32 ± 22
Afghanistan	Control	0
	Cotyledon removed	0

*SD, standard deviation.

the non-nodulating character is not a result of an inhibitor translocated from the cotyledon or plant top. Neither does the cotyledon or plant top of Trapper produce a compound which will overcome the non-nodulating character of the root of Afghanistan.

Nodulation was not observed when the plant roots were maintained at a temperature of 25 °C or 15 °C.

Our results indicate that the non-nodulating character of *P. sativum* cv. Afghanistan is unlike that described for other non-nodulating cultivars. It is not temperature dependent, as is the case with *P. sativum* cv. Iran (5); it does not involve an excreted nodulation inhibitor, as does the non-nodulating soybean (8), and nodulation is not obtained by grafting on a top of a nodulating variety, as is the case for clover (22). Furthermore, nodulation is not limited by rhizobial numbers, or by a physical barrier to entry, since infection threads are observed. Root nodule initiation begins with cell divisions in the inner cortex, in advance of the infection threads (19). The block to nodule formation seems to be at this early stage, before bacterial infection of cortical cells occurs.

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