Applied and Environmental Microbiology, Oct. 1989, p. 2532–2536 Vol. 55, No. 10
0099-2240/89/102532-05$02.00/0

Host Plant Effects on Nodulation and Competitiveness of the *Bradyrhizobium japonicum* Serotype Strains Constituting Serocluster 123

P. B. CREGAN,[1]* H. H. KEYSER,[2] and M. J. SADOWSKY[3]

Nitrogen Fixation and Soybean Genetics Laboratory, Agricultural Research Service, U.S. Department of Agriculture, BARC-West, Beltsville, Maryland 20705,[1] NifTAL Project, University of Hawaii, Paia, Hawaii 96779,[2] and Department of Soil Science and Department of Microbiology, University of Minnesota, St. Paul, Minnesota 55108[3]

Received 1 May 1989/Accepted 24 July 1989

Strains in *Bradyrhizobium japonicum* serocluster 123 are the major indigenous competitors for nodulation in a large portion of the soybean production area of the United States. Serocluster 123 is defined by the serotype strains USDA 123, USDA 127, and USDA 129. The objective of the work reported here was to evaluate the ability of two soybean genotypes, PI 377578 and PI 417566, to restrict the nodulation and reduce the competitiveness of serotype strains USDA 123, USDA 127, and USDA 129 in favor of the highly effective strain CB1809 and to determine how these soybean genotypes alter the competitive relationships among the three serotype strains in the serocluster. The soybean genotypes PI 377578 and PI 417566 along with the commonly grown cultivar Williams were planted in soil essentially free of soybean rhizobia and inoculated with single-strain treatments of USDA 123, USDA 127, USDA 129, or CB1809 and six dual-strain competition treatments of USDA 123, USDA 127, or USDA 129 versus CB1809, USDA 123 versus USDA 127, USDA 123 versus USDA 129, and USDA 127 versus USDA 129. PI 377578 severely reduced the nodulation and competitiveness of USDA 123 and USDA 127, while PI 417566 similarly affected the nodulation and competitiveness of USDA 129. Thus, the two soybean genotypes can reduce the nodulation and competitiveness of each of the three serocluster 123 serotype strains. Our results indicate that host control of restricted nodulation and reduced competitiveness is quite specific and effectively discriminates between *B. japonicum* strains which are serologically related.

Results from several studies indicate that between 50 and 90% of the nodules formed by soybeans grown in the northern midwest United States are occupied by strains of *Bradyrhizobium japonicum* that belong to serocluster 123 (8, 10, 12, 13, 16, 22). The use of the term serocluster 123 was suggested by Schmidt et al. (20) to describe the serological relationship between strains in serogroups 123, 127, and 129. Date and Decker (9) indicated that serotype strain USDA 123 possesses somatic antigens in common with both serotype strains USDA 127 and USDA 129. Strains USDA 127 and USDA 129 do not share major somatic antigens. Thus, antisera raised against USDA 123 will cross-react with cells of USDA 127 and USDA 129, whereas antisera to USDA 127 will only cross-react with cells of USDA 123 and antisera to USDA 129 will only cross-react with cells of USDA 123.

The high recovery of serocluster 123 strains from field-grown soybeans is apparently the result of the exceptional competitiveness for nodulation of this group of organisms. Ellis et al. (10) and Moawad et al. (17) documented the unique ability of indigenous serocluster 123 strains to form a large proportion of nodules even when equal populations of an inoculum strain were present in the soybean rhizosphere. In addition to their competitiveness, strains of serocluster 123 are purported to be relatively ineffective for N_2 fixation. Three different reports (2, 3, 11) indicated that when soybeans were grown in soybean rhizobium-free soil and inoculated with the serotype strain USDA 123, yields were inferior to those obtained with inoculant-quality strains. Similarly, Kvien et al. (15) indicated that whenever 50% or more of the nodules formed were occupied by inoculum strain USDA 110 or USDA 138 rather than by indigenous serogroup 123 strains, significant increases in seed yield were obtained. These reports support the contention that the displacement of serocluster 123 strains in favor of more effective *B. japonicum* strains should result in increased soybean productivity.

One approach for increasing nodulation by desirable strains is by the use of soybean genotypes on which nodulation by serocluster 123 is eliminated or substantially reduced. Reduced nodulation by this highly competitive group of organisms should allow nodulation by other strains and might provide a means of significantly increasing the nodule occupancy of an inoculum strain. To achieve this goal, we identified soybean genotypes that are nodulated poorly with strain USDA 123 (4). One of these genotypes, PI 377578, was well nodulated and effectively fixed N_2 with inoculant-quality strains such as CB1809, USDA 110, and USDA 138. When soybeans were coinoculated with both USDA 123 and the inoculant-quality strains, most nodules were formed by the latter strains. This result was in contrast to that obtained with the commonly grown soybean cultivar Williams, on which most nodules were formed by USDA 123. Thus, PI 377578 restricts the nodulation of strain USDA 123, thereby reducing the competitiveness of this strain.

More recently, we identified another soybean genotype, PI 417566 (6), that was nodulated very well with strain USDA 123 but restricted the nodulation of *B. japonicum* MN1-1c, a serocluster 123 strain. Isolate MN1-1c belongs to serogroup 127 and formed abundant nodules on the USDA 123-restricting genotype, PI 377578 (14). In addition to restricting the nodulation of isolate MN1-1c, PI 417566 also

* Corresponding author.

reduced the competitiveness of MN1-1c in favor of certain highly effective inoculant-quality strains.

The identification of PI 377578 and PI 417566 indicated the presence of very specific soybean genotype-serocluster 123 strain interactions in terms of both nodulation ability and reduced competitiveness. The discovery of these interactions raised the question of the potential interactions of the two soybean genotypes with the serotype strains USDA 123, USDA 127, and USDA 129. Therefore, the objectives of the research reported here were to determine (i) the nodulation phenotypes of USDA 123, USDA 127, and USDA 129 with PI 377578 and PI 417566, (ii) the ability of PI 377578 and PI 417566 to reduce the competitiveness of USDA 123, USDA 127, and USDA 129 in favor of the inoculum strain CB1809, and (iii) the effect of PI 377578 and PI 417566 on competitiveness among strains USDA 123, USDA 127, and USDA 129.

MATERIALS AND METHODS

A greenhouse pot experiment was conducted by using a randomized complete-block experimental design with a split-plot arrangement of treatments and four replications. Whole plots (pots) were inoculation treatments, and subplots were soybean genotypes. The potting medium was an equal-volume mixture of Monmouth fine sandy loam (clayey, mixed, mesic Typic Hapludult) soil and perlite. The soil had a very low indigenous population of soybean-nodulating bacteria (<0.4 cells/g of soil), as measured by a most-probable-number plant infection assay (21). The soil-perlite mixture was limed to neutrality with dolomite, placed in 2.2-liter pots, and wetted with 400 ml of N-free plant nutrient solution (5). Seeds of commercial *Glycine max* cv. Williams and of soybean genotypes PI 377578 and PI 417566 (originally obtained from E. E. Hartwig, Agricultural Research Service, U.S. Department of Agriculture, Stoneville, Miss.) were surface sterilized by immersion in acidified $HgCl_2$ (21) for 2 min, followed by five rinses in distilled water. Four seeds of each genotype were planted in each pot in separate holes 2 cm deep. Before being covered, the seeds (other than in the case of the uninoculated control) were inoculated with 2 ml of a stationary-phase yeast extract-mannitol (21) broth culture of 1 of 10 single- or dual-strain *B. japonicum* treatments. Single-strain treatments included USDA 123, USDA 127, USDA 129, and CB1809, and dual-strain competition treatments consisted of equal mixtures (volume to volume) of the strains indicated in Table 1. Seeds were covered with soil, and a 1-cm-thick layer of autoclaved gravel was placed on the soil surface to serve as a barrier to prevent contamination by extraneous bradyrhizobia. After emergence, plants were thinned so that one plant of each genotype remained per pot. Plants were grown in the greenhouse with natural sunlight supplemented with low-pressure sodium light to extend the photoperiod to 18 h. Day and night temperatures were maintained at 25 ± 5°C. Plants were harvested, and nodule number and dry weight were determined 36 days after planting.

TABLE 1. Competition treatments and primary antibodies used in the spot-blot analysis to determine nodule occupancy in *B. japonicum* competition experiments

Competition treatment	Primary antibodies
USDA 123 vs CB1809[a]	USDA 123 (nonspecific) and USDA 122
USDA 127 vs CB1809	USDA 123 (nonspecific) and USDA 122
USDA 129 vs CB1809	USDA 123 (nonspecific) and USDA 122
USDA 123 vs USDA 127	USDA 123 (specific: adsorbed with USDA 127 and USDA 129 antigens) and USDA 127 (specific: adsorbed with USDA 123 antigen)
USDA 123 vs USDA 129	USDA 123 (specific) and USDA 129 (specific: adsorbed with USDA 123 antigen)
USDA 127 vs USDA 129	USDA 127 (specific) and USDA 129 (specific)

[a] Strain CB1809 belongs to serogroup 122, for which USDA 122 is the antigen strain.

In the dual-strain competition treatments, nodule occupancy was determined by using spot-blot (indirect enzyme-linked immunosorbent assay) analysis. Nodules were oven dried at 65°C for 24 h, washed in a 0.1% (vol/vol) solution of Tween 80, and placed in the wells of a 96-well microdilution plate. Nodules were crushed in 100 μl of distilled water, transferred to nitrocellulose membranes, and treated as described by Ayanaba et al. (1). The antibodies used for each competition treatment are listed in Table 1. Primary antibodies with titers greater than 1,280 were produced in rabbits against the somatic antigens of strains USDA 123, USDA 127, USDA 129, and USDA 122 (serologically identical to CB1809) by the protocol of Schmidt et al. (19). Primary antibodies were used at a final concentration of 5 to 10 μg/ml, while the secondary antibody (goat anti-rabbit horseradish peroxidase [Bio-Rad Laboratories]) was used at a 1:4,000 dilution.

RESULTS

When inoculated singly with any of the four *B. japonicum* strains, Williams soybeans produced at least four times as many nodules as were present on the uninoculated Williams

TABLE 2. Mean nodule number and nodule weight of Williams, PI 377578, and PI 417566 grown in a soil-perlite mixture without inoculation or with single-strain inoculation treatments[a]

Inoculation treatment	Williams		PI 377578		PI 417566	
	Nodule no. (per plant)	Nodule wt (mg/plant)	Nodule no. (per plant)	Nodule wt (mg/plant)	Nodule no. (per plant)	Nodule wt (mg/plant)
None (uninoculated)	12 b	127 c	9 b	69 c	3 c	85 b
USDA 123	56 a	241 ab	16 b	139 bc	40 a	266 a
USDA 127	48 a	307 a	14 b	96 bc	42 a	271 a
USDA 129	59 a	251 ab	50 a	222 a	5 c	145 b
CB1809	58 a	213 b	38 a	153 ab	26 b	128 b

[a] Means within a column not followed by the same letter are different at the 5% level of probability.

TABLE 3. Nodule number and strain nodule occupancy[a] of soybean genotypes Williams, PI 377578, and PI 417566 in dual-strain competition treatments between serotype strains USDA 123, USDA 127, and USDA 129 and the inoculant-quality strain CB1809[b]

Soybean genotype	USDA 123 vs CB1809			USDA 127 vs CB1809			USDA 129 vs CB1809		
	Nodule no.	% Nodules occupied by:		Nodule no.	% Nodules occupied by:		Nodule no.	% Nodules occupied by:	
		USDA 123	CB1809		USDA 127	CB1809		USDA 129	CB1809
Williams	46 a	98 a	2 b	62 a	96 a	6 b	35 a	83 a	14 b
PI 377578	15 b	29 b	41 a	20 c	27 b	68 a	32 ab	92 a	8 b
PI 417566	44 a	98 a	7 b	36 b	98 a	3 b	17 b	5 b	89 a

[a] Strain nodule occupancies for a given genotype which add up to greater than 100% were the result of doubly occupied nodules. Occupancies which add up to less than 100% were the result of occupancy by native soybean-nodulating bacteria in the soil. In no instance did nodules formed on the uninoculated controls react with the antisera used in this experiment.

[b] Means within a column not followed by the same letter are different at the 5% level of probability.

control (Table 2). Thus, while the soil mixture was not free of soybean-nodulating bacteria, the population was well below that necessary for normal nodulation. The weight of the nodules produced by the uninoculated Williams control was also lower than that obtained with any of the four single-strain treatments. When PI 377578 was inoculated with USDA 123 or USDA 127, neither the nodule number nor the mass exceeded that of the uninoculated control. Thus, in addition to restricting the nodulation of strain USDA 123 (4), PI 377578 also restricts the nodulation of strain USDA 127. Similarly, neither the nodule number nor the mass produced by PI 417566 with USDA 129 exceeded that of the uninoculated control. Thus, PI 417566 restricts the nodulation of USDA 129, the third serotype strain that defines serocluster 123. When PI 417566 was inoculated with CB1809, the nodule number significantly exceeded that of the uninoculated control, but the nodule mass did not (Table 2). The low nodule mass suggested that PI 417566 may act to partially restrict nodulation by CB1809; however, in previous experiments we did not find this to be the case (6).

When Williams was inoculated with strain USDA 123, USDA 127, or USDA 129 in competition with CB1809, greater than 80% of the nodules formed contained the serocluster 123 strain (Table 3). This result confirms the highly competitive nature of the serocluster 123 strains on a typical North American soybean cultivar. As compared with Williams and PI 417566, PI 377578 (which restricted the nodulation of USDA 123 and USDA 127) also reduced the nodule occupancy of USDA 123 and USDA 127 when in competition with CB1809 (Table 3). As compared with Williams and PI 377578, PI 417566 (which restricted the nodulation of USDA 129) also reduced the nodule occupancy of USDA 129 when in competition with CB1809.

In the dual-strain competition treatments with the three serocluster 123 strains on Williams, the nodule occupancy data indicated that USDA 123 and USDA 129 were equally competitive and that both strains were somewhat more competitive than USDA 127 (Table 4). When PI 377578 was inoculated with USDA 123 and USDA 127 (which were both restricted by this genotype), relatively few nodules were formed and the nodule occupancy of the two strains was similar. When PI 377578 was inoculated with a restricted strain and an unrestricted strain (USDA 123 versus USDA 129 or USDA 127 versus USDA 129), the unrestricted competitor (USDA 129) formed greater than 85% of the nodules. When PI 417566 was inoculated with USDA 123 and USDA 127, neither of which was restricted by this genotype, nodule occupancy was similar to that of Williams. When PI 417566 was inoculated with an unrestricted strain and a restricted strain (USDA 123 versus USDA 129 or USDA 127 versus USDA 129), the restricted competitor (USDA 129) was recovered from only 1% of the nodules.

DISCUSSION

We have identified soybean genotypes that effectively restrict the nodulation of the three serotype strains that define serocluster 123. PI 377578 restricts the nodulation of USDA 123 and USDA 127, and PI 417566 restricts the nodulation of USDA 129. In addition to restricting the nodulation of specific serotype strains, the soybean genotypes correspondingly reduce the competitiveness of these strains in favor of the inoculant-quality strain CB1809. The two soybean genotypes also alter the competitiveness between the three serocluster 123 strains when compared with the standard North American soybean cultivar Williams. Our data clearly show a close positive relationship between restricted nodulation and reduced competitiveness. Without exception, when a host genotype restricted the nodulation of

TABLE 4. Nodule number and strain nodule occupancy[a] of soybean genotypes Williams, PI 377578, and PI 417566 in dual-strain competition treatments between serotype strains USDA 123, USDA 127, and USDA 129[b]

Soybean genotype	USDA 123 vs USDA 127			USDA 123 vs USDA 129			USDA 127 vs USDA 129		
	Nodule no.	% Nodules occupied by:		Nodule no.	% Nodules occupied by:		Nodule no.	% Nodules occupied by:	
		USDA 123	USDA 127		USDA 123	USDA 129		USDA 127	USDA 129
Williams	47 a	75 a	58 a	49 a	65 b	65 b	52 a	46 b	70 a
PI 377578	14 b	42 b	46 a	36 a	1 c	97 a	36 b	3 c	87 a
PI 417566	47 a	78 a	58 a	47 a	100 a	1 c	35 b	100 a	1 b

[a] Strain nodule occupancies for a given genotype which add up to greater than 100% were the result of doubly occupied nodules. Occupancies which add up to less than 100% were the result of occupancy by native soybean-nodulating bacteria in the soil. In no instance did nodules formed on the uninoculated controls react with the antisera used in this experiment.

[b] Means within a column not followed by the same letter are different at the 5% level of probability.

a strain, the competitiveness of that strain was also severely reduced. The very large effect of plant genotype upon competition for nodulation among strains of *B. japonicum* indicates that the soybean host must be carefully defined in studies whose objective is the assay of bradyrhizobial competition.

One aspect of the data presented here is the possible occurrence of the phenomenon whereby a strain whose nodulation is restricted by a particular host genotype functions to block nodulation by another strain that would, by itself, nodulate normally. Daitloff and Brockwell (7) reported this type of soybean-bradyrhizobium interaction in the case of cultivar Hardee, which restricts the nodulation of strain CB1809. When inoculated with other strains on Hardee, CB1809 tended to block the nodulation of the competing strain. Our data suggest that in instances in which a host genotype restricted the nodulation of a strain in the single-strain inoculation treatments (PI 377578 with USDA 123 and USDA 127; PI 417566 with USDA 129), nodulation in the dual-strain competition treatments was often reduced in relation to Williams (Table 3). This was particularly true in the USDA 123-versus-CB1809 and the USDA 127-versus-CB1809 competition treatments, in which the nodulation of PI 377578 was only about one-third that of Williams. Likewise, in the USDA 129-versus-CB1809 competition treatment, the nodulation of PI 417566 was about one-half that of Williams. Furthermore, the strain effecting the blocking, as well as the strain whose nodulation was blocked, apparently affected the degree of nodulation blocking. This was evident from the facts that the nodulation of CB1809 on PI 377578 was blocked by USDA 123 and USDA 127 (Table 3) but that these two strains appeared to demonstrate less nodulation blocking when coinoculated with USDA 129 (Table 4). Similarly, on PI 417566, USDA 129 blocked the nodulation of CB1809 but not USDA 123. We have not previously noted the phenomenon of nodulation blocking with the soybean genotypes and serocluster 123 strains with which we have been working. Studies have been initiated to further define the conditions associated with nodulation blocking.

Our data demonstrate that host control of competitiveness is quite specific and that the soybean plant can effectively discriminate between *B. japonicum* strains that are serologically related. Because soybean genotype PI 377578 (and a similar genotype, PI 371607) tended to restrict only the nodulation of *B. japonicum* strains classified as serogroup 123 (14), we had previously suggested that there was a relationship between restricted nodulation and serological classification (18). However, the results of the current work indicate that there is a more complex relationship between serology and restricted nodulation. Keyser and Cregan (14) reported that of five serogroup 127 field isolates tested, the nodulation of none was restricted by PI 377578 or PI 371607. Clearly, the restricted nodulation and reduced competitiveness of USDA 127 on PI 377578 reported here indicate that USDA 127 reacts differently than the five serogroup 127 strains mentioned above. The interaction of PI 417566 with isolates of serogroup 127 is also inconsistent. This genotype does not restrict the nodulation of USDA 127 but does restrict the nodulation of isolate MN1-1c, which belongs to serogroup 127 (6). These findings suggest the necessity to further define the possible relationship between the serological classification of *B. japonicum* and the ability of a strain to nodulate a particular soybean genotype.

ACKNOWLEDGMENTS

We thank Pam Prigg and Deborah Thibeau for excellent technical assistance during the course of this study. We also thank Jeff Fuhrmann and Arvind Bhagwat for careful reading of the manuscript.

This research was supported in part by U.S. Department of Agriculture competitive grant 88-37231-4003.

LITERATURE CITED

1. **Ayanaba, A., K. D. Weiland, and R. M. Zablotowicz.** 1986. Evaluation of diverse antisera, conjugates, and support media for detecting *Bradyrhizobium japonicum* by indirect enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. **52:** 1132–1138.
2. **Caldwell, B. E., and G. Vest.** 1970. Effect of *Rhizobium japonicum* strains on soybean yields. Crop Sci. **10:**19–21.
3. **Chamber Perez, M. A., R. Orive Echevarrieta, F. J. Temprano Vera, and G. Lavandera Gonzalez.** 1983. Seleccion de raza de *Rhizobium japonicum* para la produccion de inoculantes para soja. Eurosoya **1:**21–25.
4. **Cregan, P. B., and H. H. Keyser.** 1986. Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA 123 in soybean. Crop Sci. **26:**911–916.
5. **Cregan, P. B., and H. H. Keyser.** 1988. Influence of *Glycine* spp. on competitiveness of *Bradyrhizobium japonicum* and *Rhizobium fredii*. Appl. Environ. Microbiol. **54:**803–808.
6. **Cregan, P. B., H. H. Keyser, and M. J. Sadowsky.** 1989. Soybean genotype restricting nodulation of a previously unrestricted serocluster 123 bradyrhizobia. Crop Sci. **29:**307–312.
7. **Daitloff, A., and J. Brockwell.** 1976. Ecological studies of root-nodule bacteria introduced into field environments. 4. Symbiotic properties of *Rhizobium japonicum* and competitive success in nodulation of two *Glycine max* cultivars by effective and ineffective strains. Aust. J. Exp. Agric. Anim. Husb. **16:**514–521.
8. **Damirgi, S. M., L. R. Frederick, and I. C. Anderson.** 1967. Serogroups of *Rhizobium japonicum* in soybean nodules as affected by soil types. Agron. J. **59:**10–12.
9. **Date, R. A., and A. M. Decker.** 1965. Minimal antigenic constitution of 28 strains of *Rhizobium japonicum*. Can. J. Microbiol. **11:**1–8.
10. **Ellis, W. R., G. E. Ham, and E. L. Schmidt.** 1984. Persistence and recovery of *Rhizobium japonicum* inoculum in a field soil. Agron. J. **76:**573–576.
11. **Ham, G. E.** 1980. Interactions of *Glycine max* and *Rhizobium japonicum*, p. 289–296. *In* R. J. Summerfield and A. H. Bunting (ed.), Advances in legume science. Royal Botanical Gardens, Kew, United Kingdom.
12. **Ham, G. E., L. R. Frederick, and I. C. Anderson.** 1971. Serogroups of *Rhizobium japonicum* in soybean nodules. Agron. J. **63:**69–72.
13. **Kapusta, G., and D. L. Rouwenhorst.** 1973. Influence of inoculum size on *Rhizobium japonicum* serogroup distribution frequency in soybean nodules. Agron. J. **65:**916–919.
14. **Keyser, H. H., and P. B. Cregan.** 1987. Nodulation and competition for nodulation of selected soybean genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. Appl. Environ. Microbiol. **53:**2631–2635.
15. **Kvien, C. S., G. E. Ham, and J. W. Lambert.** 1981. Recovery of introduced *Rhizobium japonicum* strains by soybean genotypes. Agron. J. **73:**900–905.
16. **Miller, R. H.** 1979. Field survey of the distribution of *Rhizobium japonicum* serogroups in Ohio, p. 2. *In* R. W. Weaver and D. A. Zuberer (ed.), Proceedings of the 7th North American Rhizobium Conference. Texas A&M University, College Station.
17. **Moawad, H. A., W. R. Ellis, and E. L. Schmidt.** 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. Appl. Environ. Microbiol. **47:**607–612.
18. **Sadowsky, M. J., R. E. Tully, P. B. Cregan, and H. H. Keyser.** 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup

123 and its relation to genotype-specific nodulation of soybean. Appl. Environ. Microbiol. **53**:2624–2630.
19. **Schmidt, E. L., R. O. Bankole, and B. B. Bohlool.** 1968. Fluorescent antibody approach to the study of rhizobia in soil. J. Bacteriol. **95**:1987–1992.
20. **Schmidt, E. L., M. J. Zidwick, and H. H. Abede.** 1986. *Bradyrhizobium japonicum* serocluster 123 and diversity among member isolates. Appl. Environ. Microbiol. **51**:1212–1215.
21. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. IBP handbook. 15. Blackwell Scientific Publications, Ltd., Oxford.
22. **Weber, D. F., H. H. Keyser, and S. L. Uratsu.** 1989. Serological distribution of *Bradyrhizobium japonicum* from U.S. soybean production areas. Agron. J. **81**:786–789.