

THE EFFECT OF SIMAZINE, KINETIN, AND *RHIZOBIUM PHASEOLI* ON LEGUME NODULATION AND MORPHOGENESIS IN *PHASEOLUS VULGARIS* L., cv. "RED KIDNEY"^{1, 2}

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ABSTRACT

The growth responses of *Phaseolus vulgaris* L., cv. "Red Kidney" plants to simazine at 5×10^{-5} M and 5×10^{-6} M, kinetin at 9×10^{-7} M and 9×10^{-8} M, and the presence or absence of *Rhizobium phaseoli* ATCC14482, in factorial combination, were measured. The plants were analyzed with respect to the lengths of the primary-through-quaternary leaves, stem height, fresh and dry weights, number of flowers, and number and size of nodules. An analysis of variance showed that simazine significantly depressed all parameters at both concentrations. Kinetin did not affect the lengths of the primary-through-tertiary leaves, but depressed the quaternary, and also decreased dry weights at the higher concentration. Kinetin had no effect on stem height; it decreased flowering, but enhanced nodulation. The presence of *Rhizobium* had a significant effect only on flowering and nodulation, increasing both.

INTRODUCTION

The importance to agriculture of the nitrogen fixation that occurs in consequence of the symbiotic association of species or strains of *Rhizobium* with species of legumes is today well known. The rhizobia occur in the soil and certain strains thereof are able to infect the roots of certain legumes, causing a localized hyperplasia, or nodulation, to occur. Nitrogen fixation occurs in the nodules (if the infective rhizobial strain is an "effective" one for the legume in question), which is why the process of nodule formation is of such interest.

Stewart (1966) and Dixon (1969) have reviewed the history of studies on this symbiotic relationship, beginning with the 1888 studies of Hellriegel and Wilfarth, who proved the necessity of nodulation for nitrogen fixation, and the isolation by Beijerinck of *Rhizobium* the same year.

Allen and Allen (1950) have reviewed nodule morphogenesis and, more recently, Nutman (1963) has described the three stages of nodule development as being: (1) infection of the root by penetration of the bacteria and consequent release of indole-acetic acid (IAA); (2) initiation and organization of the nodule; and (3) the intracellular phase, with bacteroid forms (irregularly shaped, but viable cells) being prominent.

Various chemical and biological factors have been tested to determine their influence on legume nodulation. Both Darbyshire (1966) and Munns (1968b) have observed that nitrate salts inhibited nodulation in *Trifolium glomeratum* and *Medicago sativa*.

IAA was found to be essential for *Rhizobium* infection and nodule initiation (Tanner and Anderson, 1964). Inhibition of nodulation by combined nitrogen

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occurred because the rhizobia reduced applied nitrate to nitrite, which catalytically destroyed IAA (Dixon, 1969). Ammonia, urea, and glycine acted similarly (see Cartwright and Snow, 1962, for effect of urea).

The specificity of *Rhizobium* species for strains of their appropriate legumes has been studied. On the basis of their results, Peters and Alexander (1966) hypothesized that there were root-exudate factors which stimulated their homologous rhizobia. Valera and Alexander (1965) studied the influence of alfalfa-seed extract and coconut water on the symbiosis. Alfalfa-seed extract, when added to excised, cultured roots, hastened the formation of nodules, while coconut water inhibited root growth.

Simazine (2-chloro-4, 6-bis (ethylamino)-s-triazine), used as an herbicide, is able to alter nitrogen metabolism in higher plants (Beevers and Hageman, 1969). Its primary effect, however, seems to be on photosynthesis (Audus, 1964; Moreland, 1967), either on CO₂ fixation in light and/or on electron flow in photosynthesis. Saburova and Petunova (1965) showed that simazine inhibited both growth and development of oats and corn and also caused chlorosis to occur, concluding that the effect of the chemical on chlorophyll *per se* is secondary to the disturbance of carbohydrate metabolism. The s-triazine herbicides in general, including simazine, are not toxic to rhizobia (Kasziubiak, 1966).

There have been few investigations into the effect of cytokinins on nodulation. Arora *et al.* (1959) were able to induce pseudonodules in tobacco root only after a second application of kinetin (6-furfuryl-aminopurine) (0.2 mg/1.) in the axial areas of lateral rootlets. Torrey (1961) found that kinetin induced the division of mature tetraploid pea-root cortical cells. He suggested that the rhizobia may contribute a kinetin-like factor and so stimulate cell division in the root cortex. Studies of this nature are useful in comprehending the underlying pattern of growth-regulator production prerequisite to or concomitant with infection and nodulation.

Thus, while it is known that IAA functions in nodule formation, cytokinins have not hitherto been clearly shown to be implicated, to our knowledge, in this process. Also, the effect of simazine on nodulation as such is not certain. Therefore, the primary purpose of our experiments was (1) to see if either or both kinetin and simazine had an effect on nodulation and on several other growth and development parameters in *Phaseolus vulgaris* L., cv. "Red Kidney", with or without inoculation with *Rhizobium phaseoli*, and (2) to determine whether there was any interaction between the three variables in their effect on the parameters measured. These parameters were height, dry weights of the tops of the plants, lengths of primary-through-quaternary leaves, the number of flower buds formed, and the number of nodules formed.

MATERIALS AND METHODS

Seeds of *Phaseolus vulgaris* L., cv. "Red Kidney" (Bolgiano Seed Co.), were surface-sterilized by successive two-minute immersions in a detergent, 80 percent ethanol and 5 percent sodium-hypochlorite solution, followed by distilled-water rinsings to remove all traces of the disinfectants. The seeds were then planted *en masse* in new horticultural-grade vermiculite. Single seedlings were transplanted into more vermiculite when the seedlings had partially expanded primary leaves. No fertilizer was given the plants, and they were watered with distilled water as required. The plants were maintained under greenhouse conditions at 25°C during the 11 hr day and at 20°C during the 13 hr night. No deficiency symptoms were seen in the four-week experimental period.

The bean plants were then divided into groups or sets in which the following factors were applied in all combinations: simazine added at 5×10^{-5} M, 5×10^{-6} M, and zero concentrations, kinetin added at 9×10^{-7} M, 9×10^{-8} M, and zero con-

centrations, and an inoculum of *Rhizobium phaseoli* ATCC 14482 being either present or absent (Table 1). The *Rhizobium* inoculum was cultured in a yeast-extract-mannitol broth consisting of, per liter: mannitol, 10g; K₂HPO₄, 0.5g; MgSO₄, 0.2g; NaCl, 0.10g; and yeast extract, 100 ml.

TABLE 1

Experimental sets of ten Phaseolus vulgaris (red kidney) plants each receiving various levels of Simazine, Kinetin, and Rhizobium inoculation

Kinetin Concn.	Inoculated Simazine Concn.			Uninoculated Simazine Concn.		
	0	5×10 ⁻⁶ M	5×10 ⁻⁵ M	0	5×10 ⁻⁶ M	5×10 ⁻⁵ M
0	A ₁ B ₁	A ₂ B ₁	A ₃ B ₁	A ₁ B ₁	A ₂ B ₁	A ₃ B ₁
9×10 ⁻⁸ M	A ₁ B ₂	A ₂ B ₂	A ₃ B ₂	A ₁ B ₂	A ₂ B ₂	A ₃ B ₂
9×10 ⁻⁷ M	A ₁ B ₃	A ₂ B ₃	A ₃ B ₃	A ₁ B ₃	A ₂ B ₃	A ₃ B ₃

The growth medium was autoclaved for 15 minutes at 20 lb pressure in 300 ml quantities and then was inoculated with 15 ml of a 3-day culture. The yeast-extract-mannitol medium containing *Rhizobium* was incubated at 28°C for two days. Population growth was studied by plating out dilutions of 10⁻⁶ to 10⁻¹⁰ on yeast-mannitol agar. A Quebec colony counter was used in direct counting. Five ml of a 10⁷-cell-per-ml yeast-mannitol broth was poured over the vermiculite of those pots due to receive the inoculum and watered in. Microscopic examination of the broth, before the rhizobia were applied, assured that short rods were present. The simazine and kinetin solutions (one 5 ml portion each) were applied in similar fashion to the surface of the vermiculite where called for.

Four weeks after treatment, the plants were harvested and tabulations made of leaf lengths, stem heights, number of flower buds, and the size and number of nodules, per set. Dry weights of the tops (i.e., above the first secondary roots) of the specimens were also obtained, after having been in the drying oven at 90°C for 24 hours. Analyses of variance were performed comparing the above parameters for the inoculated and uninoculated groups separately.

Nodules were collected randomly from the bean plants and cleansed in 1 g of mercuric chloride per 500 ml of water, which was followed first by a 5-ml washing of 1.0 N hydrochloric acid and then by several rinses in sterile distilled water. The nodules were crushed and then plated on yeast-mannitol agar. Rhizobia were detected microscopically.

Bacteroid forms were identified by observing material from nodules crushed between two microscopic slides. A loopful of the crushed nodular material was smeared on a clean slide and stained with crystal violet. Reisolation was effected by placing a loopful of the crushed specimen in a tube of yeast-extract-mannitol broth. Fermentation of sugars and reactions with the litmus milk were performed to identify the presence of *Rhizobium phaseoli*.

Several nodules from each treatment were also fixed in formalin-acetic acid and prepared by paraffin embedding for histological examination. Sections were stained with safranin 0 and fast green.

The leaf lengths, stem heights, dry weights of the tops, number of flower buds, and number and size of nodules per group were compared and evaluated statistically, the groups with *Rhizobium* and the groups without *Rhizobium* being

analyzed separately, using a 2×2 factorial analysis-of-variance test (Winer, 1962). Each of the F values was tested at the .05 significance level, using the tables of Arkin and Colton (1966).

RESULTS

The effects of the different treatments varied for the different parameters measured. These results are presented below, summarized for each parameter: leaves, stem height, dry weights, number of flower buds, and nodule formation.

Leaves

The primary and secondary leaves did not respond to the presence of *Rhizobium*, which had no significant effect on their growth (Table 2). Kinetin also had a statistically insignificant influence, whereas simazine significantly decreased leaf length in both primary and secondary leaves.

TABLE 2
Average length (cm.) of leaves of the inoculated and uninoculated groups of *Phaseolus vulgaris* L. (red kidney)

Treatment ^{1, 2}	Primary Leaves		Secondary Leaves		Tertiary Leaves		Quaternary Leaves	
	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated
A ₁ B ₁	5.75	6.32	7.20	6.92	7.03	6.37	3.85	2.50
A ₁ B ₂	5.84	6.71	6.85	4.55	5.60	3.93	1.72	1.02
A ₁ B ₃	5.34	6.00	6.97	5.91	6.68	4.66	2.33	6.83
A ₂ B ₁	5.80	4.09	6.81	3.44	6.96	2.58	5.12	0.00
A ₂ B ₂	5.06	5.47	6.63	6.13	6.81	4.79	1.33	8.80
A ₂ B ₃	5.08	3.66	6.83	3.39	7.77	1.82	3.84	7.09

¹A₁=simazine (absent)

A₂=simazine (5×10^{-6} M)

A₃=simazine (5×10^{-5} M)

B₁=kinetin (absent)

B₂=kinetin (9×10^{-8} M)

B₃=kinetin (9×10^{-7} M)

²Treatments A₃B₁, A₃B₂, and A₃B₃ have been omitted from this table since the 5×10^{-5} M simazine caused the death of all leaves.

Tertiary and quaternary leaves treated with rhizobia were significantly longer (Table 2). Application of simazine at 5×10^{-6} M permitted growth nearly as great as in those groups not treated with simazine, in contrast to the results obtained at the concentration of 5×10^{-5} M, where growth was nil. In fact, the higher concentration of simazine was ultimately lethal to the plant. Kinetin did not significantly affect the development of the tertiary leaves, but seemed to depress growth of the quaternary leaves at 9×10^{-8} M concentration. In the higher concentration (9×10^{-7} M) of kinetin, the quaternary-leaf length approached the mean.

Height

Stem height was decreased by simazine (5×10^{-5} M). The presence or absence of kinetin and of *Rhizobium*, irrespective of the presence or absence of simazine, resulted in no statistically significant enhancement or depression of development (Table 3). Even so, a trend toward decreased stem height is apparent with increasing kinetin concentration, especially at the higher simazine concentration.

Dry Weights

Dry weights, both of the groups which were exposed to *Rhizobium* and of those

which were unexposed, did not differ significantly (Table 3). However, the effects of kinetin and simazine were significant at the .05 level. Plants treated with the lower level of kinetin resembled those lacking the hormone, but the higher level of kinetin produced an actual decrease in dry weight. The influence of the simazine is marked. Specimens treated with 5×10^{-5} M simazine were very much reduced in dry weight as compared to those treated with 5×10^{-6} M simazine, which, in turn, were lower than the controls.

Flower Buds

The number of flower buds was greater in the *Rhizobium*-inoculated groups (Table 3) than in those not receiving it. In sets treated with simazine at either concentration, and in sets treated with kinetin at either concentration, the count was less than in sets not receiving either substance (Table 3).

TABLE 3

Effects of treatments with Simazine, Kinetin, and Inoculation with Rhizobia on Height, Dry Weight, Number of Flower Buds, and Number of Nodules on Plants of Phaseolus vulgaris L. (red kidney)

Treatment with Kinetin and Simazine ¹	Average Height (cm)		Average Dry Weight (gm)		Average Number of Flower Buds		Average Number of Nodules	
	Inocu- lated	Uninocu- lated	Inocu- lated	Uninocu- lated	Inocu- lated	Uninocu- lated	Inocu- lated	Uninocu- lated
A ₁ B ₁	17.2	19.4	0.487	0.54	5	3	14	10
A ₁ B ₂	16.7	17.6	0.515	0.53	2	1	43	5
A ₁ B ₃	15.9	16.7	0.455	0.43	3	2	67	2
A ₂ B ₁	17.1	17.7	0.417	0.34	2	0	46	0
A ₂ B ₂	18.0	18.9	0.353	0.46	2	1	28	0
A ₂ B ₃	16.0	18.0	0.265	0.22	3	0	45	0
A ₃ B ₁	13.5	15.3	0.146	0.16	0	0	0	0
A ₃ B ₂	12.7	11.7	0.148	0.11	0	0	0	0
A ₃ B ₃	10.3	11.9	0.142	0.12	0	0	0	0

¹Concentrations as in Table 1.

Nodules

Both the simazine and the kinetin were statistically significant for nodule number, as was the presence of the rhizobia. Simazine at high concentrations inhibited nodulation, while at low concentrations, it only reduced the nodulation slightly. Kinetin increased nodulation at increasing levels (Table 3).

Sectioning of nodules from plants revealed the cytological relationships of the bacterium to the surrounding leguminous cells. The nodules in both uninoculated and inoculated groups possessed the inner-vacuolar-type arrangement of infected tissue, consisting of an outer epidermal layer, a cortical layer, and a central vacuolation. In groups treated with kinetin (9×10^{-8} M), cells of the cortical area seemed enlarged, whereas this same type of tissue was much reduced in those plants treated with simazine (5×10^{-6} M). No nodules were formed in samples treated with simazine in high concentrations. It should also be noted that, in the uninoculated groups, nodules formed only in the control group and in sets treated with kinetin.

DISCUSSION

It is known (Letham, 1967) that cytokinins retard root, stem, and leaf growth of plants grown under natural photoperiods. Humphries (1958), for example, reported that kinetin depressed the rate of leaf growth, both in normal light-grown plants and with leaf disks. Such growth depression may be the consequence of kinetin-gibberellin antagonism (Letham, 1967).

In our own work, the primary-through-tertiary leaves, in both the inoculated and uninoculated groups, were not influenced by kinetin to a statistically detectable degree. Only by the time when quaternary leaves had appeared was kinetin exerting a significant depressing effect, although Table 2 indicates that kinetin at $9 \times 10^{-7}M$, with or without simazine, depresses leaf expansion. At $9 \times 10^{-8}M$ concentration of kinetin, there might even be a mild stimulation. However, Kulaeva (1962) has demonstrated, using *Nicotiana rustica*, that exogenously applied kinetin is active only on excised leaves; in intact plants, the roots supplied substances to the leaves which counteracted the activity of kinetin, an effect not duplicated in the aerial portions of the kinetin-treated leaves. Holm and Key (1969) came to the same conclusion. Nevertheless, our intact plants showed a kinetin effect on the quaternary-leaf lengths.

Cytokinins applied to leaves and possibly to stems are not appreciably translocated (Letham, 1967). The delay in response by our bean plants to the kinetin applied to the potting medium may therefore be a reflection of a slow uptake by roots. Cytokinins also seem to move more readily in a basipetal, rather than acropetal, direction (Letham, 1967).

Simazine at the higher concentration of $5 \times 10^{-5}M$ (A_3 in Table 1) destroyed all the leaves, which dried up and become unmeasurable. At the lower concentration ($5 \times 10^{-6}M$), while there was a chlorosis, leaf length was not significantly altered. The data in Table 2 indicate that the effect of simazine was greater in the uninoculated groups.

The presence or absence of rhizobia had not significant effect until the time of appearance of the tertiary and quaternary leaves. Conceivably, the longer lengths of the tertiary and quaternary leaves in the inoculated groups represents the beginnings of the effects of nitrogen fixation.

Stem height was not significantly affected by the application of kinetin. This is rather surprising, considering the known retarding effects on growth by cytokinins (Letham, 1967). To be sure, the experiment was only pursued for four weeks. Assuming a slow rate of translocation of cytokinins, as suggested above, it seems possible that a significant effect would have appeared in due course. Even so, some reduction in height with increasing kinetin concentration can be seen, especially in the inoculated group. *A priori* one would have expected the presence of rhizobia, and as a consequence, nitrogen fixation, to have resulted in an increase in plant height, but this did not occur. Simazine, however, caused a significant reduction in the height of plants.

Variable results have been published as to the effect of kinetin on dry weight and fresh weight. Miller *et al.* (1955) found remarkable cell division of tobacco callus as a result of addition of kinetin and IAA, though neither fresh weight nor dry weight increased with application of the growth hormone to *Raphanus sativus* L. Katsumi (1962) stated that kinetin showed no significant effect on fresh weight of etiolated pea-stem sections. In our own experiment, kinetin at $9 \times 10^{-7}M$ caused a decrease in dry weight.

Simazine, if applied to crops either in a post-emergence or pre-emergence manner, prevents the germination and growth of broadleaved herbs. If it is applied in quantities greater than 0.5 ppm, reduction in dry weight may also result. Moreover, if a particular species is especially susceptible to the herbicide, injury to the leaf may also occur (Crafts, 1961). Our plants, treated with $5 \times 10^{-5}M$ and $5 \times 10^{-6}M$ of simazine, showed this reduction in dry weight. As evidence of simazine's phytotoxic nature, leaves in groups treated with $5 \times 10^{-5}M$ simazine became chlorotic. Moreland (1959), Fink and Fletchall (1967), and Ries *et al.* (1967) have all reported on the various aspects of the phytotoxicity of simazine.

Kinetin ($9 \times 10^{-8}M$) slightly stimulated flower buds in the sets treated with *Rhizobium* in this study, but generally those sets receiving kinetin (B_2 and B_3 in Table 1) had fewer buds than did those which did not (B_1). This is contrary to

reports by others (e.g., Michniewicz and Kamienska, 1964 and 1965), who have reported flour-bud induction by cytokinins. See also Mullins (1967).

The suppression of flower-bud initiation may be accounted for by the generally depressing effect of simazine on carbohydrate metabolism, through its effect on photosynthesis (Hilton *et al.*, 1963). Another possible explanation for this is simazine's well-documented effect on nitrogen metabolism, (e.g., Fink and Fletchall, 1967; Ries, *et al.*, 1967; and Tweedy and Ries, 1967). Sublethal amounts of simazine both enhance nitrate assimilation by increasing nitrate reductase levels and increase the protein content of some species, including legumes (pea). It is conceivable that this treatment might enhance vegetative growth and delay the onset of flowering, although the above-named workers have not found any reduction in yield of seeds. In this connection, it is interesting that Lips and Roth-Bejerano (1969) found that kinetin plus gibberellic acid (10 ppm and 20 ppm, respectively) also induce nitrate reductase in tobacco in the dark.

Kinetin causes an acceleration of cell division. This growth regulator *in vitro* can initiate the growth of buds and roots (Letham, 1967). Heide (1965) tested the effects of the cytokinin 6-benzylaminopurine on bud formation in *Bryophyllum* and concluded that, with the addition of this cytokinin, budding was enhanced. Because the activity of 6-benzylaminopurine is similar to kinetin, similar responses, such as those in nodulation, should be expected with kinetin. It is also believed that root exudates may produce an effect similar to kinetin, since the root is a site of cytokinin synthesis (Sutton and Itai, 1967). Kinins are believed to contribute to the formation of nodules (Arora *et al.*, 1959). Although substances such as polygalacturonase (Barrios, *et al.*, 1963) and leghemoglobin (Virtanen, 1947) have also been hypothesized as the unknown morphogenetic factor initiating nodulation, Lillich and Elkan (1968; see also Dixon, 1969) could find no enzyme in *Rhizobium*, nor was the enzyme induced with pectin or galactose. In addition, root extracts and exudates of *Glycine max* (soybean) did not show any higher enzyme activity after inoculation.

In the sets treated with *Rhizobium* in our experiment, kinetin significantly increased nodulation. Arora *et al.* (1959) observed that pseudonodules occurred on tobacco *in vitro* when treated with kinetin. They also state that such false nodules may also occur naturally under conditions where quantities of growth substances are released through the action of microorganisms present in the vicinity of the root systems. These growth substances or cytokinins may be excreted from the roots. In our experiment, we may well be seeing a synergism between the microorganism and the exogenous supply of cytokinin, supplementing that normally present.

Nodulation at the 5×10^{-5} M-simazine level was inhibited. However, root nodules were not absent in sets treated at concentration 5×10^{-6} M. This may be the level at which the plant and microsymbiont are able to overcome the toxic effects of the chemical.

Sections of nodules obtained using the paraffin method did not allow observation of intracellular bacteria. Literature (e.g., Goodchild and Bergersen, 1966) substantiates the need for electron microscopy to determine the position of these microorganisms. However, simazine affected the root area by decreasing the number of cells of the cortical region and by distorting the shapes of the cells in the roots. Cell division seemed to be stimulated by kinetin at 9×10^{-8} M. Nodules of all groups possessed distinct mature central vacuoles (hollow regions) except the nodules subjected to simazine at low concentration, where they were not well developed.

Just how the simazine reduces nodulation, at least at the higher concentration of 5×10^{-5} M, is not certain. As Dixon (1969, p. 146) points out, combined nitrogen (*viz.*, amino acids, nitrite, nitrate), especially nitrate (Munns, 1968b), inhibits

legume-root nodulation. Apparently, this is not an effect on the rhizobia, but a result of the destruction of IAA by nitrite. Cited above was the enhancing effect of sublethal simazine on nitrogen metabolism, especially the enhancement of nitrate reductase synthesis (Ries, *et al.*, 1967; Tweedy and Ries, 1967). Perhaps the simazine stimulates the formation of greater-than-usual amounts of nitrite by the extra nitrate reductase, which nitrite then catalyzes the destruction of the IAA necessary for nodulation (Dixon, 1969).

Though it is evident that the presence of *Rhizobium* induced nodulation, chance contamination occurred in groups not treated with the bacterium. This nodulation in uninoculated sets was at first thought possibly to have been induced solely by kinetin, but nodules were evident in the uninoculated control group also, and the differences were not statistically significant. Later, histological studies revealed that contamination may have been caused by a bacterium.

In sum, the most important of our findings were that kinetin, contrary to published reports (Letham, 1967), inhibits flowering. However, it does enhance nodulation, apparently facilitating the positive effect of rhizobia in this process. Kinetin also depresses the growth of attached leaves, after a considerable time lag, as others have found (Letham, 1967). Finally, simazine significantly depresses the nodulation process.

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