EFFECTS OF RESPIRATION INHIBITORS ON ACCUMULATION OF RADIOACTIVE PHOSPHORUS BY ROOTS OF LOBLOLLY PINE

PAUL J. KRAMER

Received August 5, 1950

The close correlation between mineral nutrient accumulation and metabolic activity of roots has become well established during the past two decades (**3**). This correlation is based largely on observations of the effects of aeration, temperature, and carbohydrate supply of the roots on accumulation of nutrients. It would be expected that substances inhibiting root respiration would inhibit the accumulation of minerals. In one of the few studies involving the use of respiration inhibitors MACHLIS (**7**) reported that cyanide and azide inhibited two-thirds of the respiration of barley roots and completely inhibited accumulation of bromide ion, and there was indirect evidence that potassium accumulation was inhibited. Iodoacetate and malonic acid also inhibited both respiration and salt accumulation of barley roots. LATIES (**6**) likewise reported that iodoacetate, malonic acid, and fluoride inhibit respiration of barley roots, but he did not study their effects on salt accumulation.

No studies of this sort have been made on roots of woody species. A series of experiments was therefore performed to find whether or not respiration inhibitors reduce mineral accumulation in loblolly pine (*Pinus taeda* L.) roots as they do in barley roots. An attempt was also made to learn whether or not mycorrhizal roots react in the same manner as non-mycorrhizal roots. Since mycorrhizae represent a complex structure, consisting of both root tissue and fungal hyphae, it seemed possible that they might differ physiologically from those roots which have no fungus associated with them.

Experiments with respiration inhibitors

Roots were obtained from potted loblolly pine seedlings growing out of doors and from seedlings growing in a clearing in the forest. The root tips and segments bearing mycorrhizae were freed of foreign matter by gently brushing with a camel's hair brush in a shallow tray of distilled water. A total of 48 segments, each at least 2 cm. in length and bearing a healthy, growing tip, were selected and separated into four groups for the comparison of the various inhibitors. Since it was impossible to find 48 root tips of the same diameter, the tips were first divided into 12 groups, each containing four roots of approximately the same dimensions. One root from each group was then used in each treatment so the various sizes of roots were uniformly distributed among the four treatments. Roots bearing mycorrhizal branches were cut into segments 1 cm. in length and selected

for uniformity in size and number of mycorrhizal branches in the same manner as the root tips. The mycorrhizal branches used in experiment 1 were small, usually consisting of only two to four tips; but those used in experiment 2 consisted of large coralloid masses. The root segments were placed in distilled water contained in open specimen dishes. Those to be treated with respiration inhibitors were placed in the desired concentrations of these inhibitors for an hour to allow the inhibitors to penetrate the roots and reduce metabolic activity before the roots were placed in solutions containing phosphorus. They were then transferred to other dishes containing the same concentration of inhibitor, plus sufficient P^{32} to produce an activity of about 300 microcuries per liter. The control segments were placed in distilled water during the conditioning period, then transferred to distilled water plus P^{32}, adjusted to the desired pH. The concentrations of inhibitors used were based on observations of KELLY (**4**), LATIES (**6**), and STENLID (**8**). Stenlid observed that sodium azide completely inhibits respiration of barley and wheat roots at pH 4.5, but produces no inhibition at pH 7.0. The writer likewise found in preliminary experiments that azide inhibits accumulation of P^{32} to a greater extent at pH 4 to 5 than at higher pH values. LATIES (**6**) found inhibition of respiration of barley roots by malonic acid and iodoacetic acid to be much greater at pH 4 to 5 than at higher values. He attributed this to better penetration of the inhibitors in more acid solutions. The pH of the solutions used in these experiments were therefore adjusted by adding dilute NaOH to the malonic acid and dilute HCl to the other solutions. Although the solutions were left unbuffered to avoid adding phosphate in addition to that in the radioactive solutions, little change in pH occurred during the experiments.

The roots were left in the solutions in shallow, open dishes for three hours, since Laties' data indicate that maximum inhibition of respiration usually occurs within three hours. The temperature varied from 25 to 30° C in various experiments. After three hours the roots were removed, rinsed for 30 seconds in distilled water, rinsed for a few seconds in 0.001 *N* H_3PO_4, and again rinsed in distilled water. Rinsing in acid was intended to remove adsorbed radioactive phosphate ion from the surface of the roots by exchange with that in the dilute acid. The terminal centimeter of each root tip was cut off and retained for counting. The root segments and tips were allowed to dry overnight at room temperature, and the activity of each segment was then measured with a Geiger-Muller counter. The results of two experiments with three inhibitors at different pH values are summarized in table I. Inspection of the data for experiment 1 indicates that malonic acid slightly inhibited accumulation of P^{32}, but a statistical analysis of the data revealed that this apparent small inhibition was insignificant. The differences between mycorrhizal roots and nonmycorrhizal root tips proved to be significant, indicating that accumulation by mycorrhizal roots is inhibited to a lesser extent than it is in nonmycorrhizal roots. The results of experiment 2 indicate that the inhibiting effect of azide is decreased at a higher

TABLE I

EFFECT OF RESPIRATION INHIBITORS ON ACCUMULATION OF P^{32} BY PINE ROOTS. RESULTS ARE AVERAGES OF 12 SAMPLES IN EACH TREATMENT.

Treatment	Root tips		Mycorrhizal segments	
	Counts/min.	Activity as % of control	Counts/min.	Activity as % of control
Experiment 1 pH 4.7–4.8				
Control	81,713	...	77,306	...
0.025 M Malonic Acid.....	77,330	94.6	67,405	87.2
0.001 M Sodium Azide	308	0.4	965	1.2
0.005 M Sodium Fluoride ..	986	1.2	1,620	2.1
Experiment 2 pH 5.7–5.8				
Control	20,017	...	117,235	...
0.025 M Malonic Acid.....	30,840	154	151,940	130
0.001 M Sodium Azide	3,796	19	44,845	38
0.005 M Sodium Fluoride ..	23,870	119	175,516	150

pH value, but accumulation by nonmycorrhizal roots is still inhibited more than it is in mycorrhizal roots. More surprising is the observation that malonic acid and sodium fluoride increase accumulation of P^{32} at pH 5.7–5.8. This experiment was repeated, and similar results were obtained.

EFFECTS OF pH ON INHIBITION OF ACCUMULATION

In the course of the experiments it became clear that pH has a very marked effect on the inhibitory action of sodium azide, corroborating the observations of STENLID (8) on wheat and barley roots. Some of the data obtained with pine roots are summarized in table II. These data not only show that decreasing inhibition occurs with increasing pH, but that accumulation of P^{32} is consistently inhibited more in root tips than in mycorrhizal roots. The amount of P^{32} accumulated, expressed as a percentage of the control, is two to three times as great in the mycorrhizal roots as in the

TABLE II

EFFECTS OF pH ON EXTENT OF INHIBITION BY 0.001 M SODIUM AZIDE OF ACCUMULATION OF P^{32} BY PINE ROOTS. RESULTS ARE BASED ON AVERAGES OF ALL SAMPLES COUNTED.

Treatment	No. of samples	Root tips		Mycorrhizal segments	
		Counts/min.	Activity as % of control	Counts/min.	Activity as % of control
pH 4.7–4.8	12	308	0.4	965	1.2
pH 5.0	8	458	2.4	2,711	6.2
pH 5.5	8	115	12.0	796	39.0
pH 5.7–5.8	12	2,669	38.0	62,648	87.0

nonmycorrhizal root tips. While the actual values are very small at pH 4.7–4.8, the difference between them is statistically significant.

The results of these experiments support the conclusions of Stenlid and Laties concerning the importance of pH as a factor affecting the degree of inhibition produced by various chemicals. Reference to table I shows that at pH 4.7–4.8 azide and fluoride strongly inhibited the accumulation of phosphorus, but at pH 5.7–5.8 azide inhibited it to a lesser extent and fluoride and malonic acid actually stimulated accumulation. The slight, apparent decrease in accumulation of phosphorus in the presence of malonic acid observed in experiment 1, table I, is not statistically significant. Experiment 2 was repeated, with similar results; hence the increased accumulation of phosphorus in the presence of malonic acid or of fluoride at pH 5.7–5.8 appears to be adequately substantiated. Laties' data indicate that 0.01 M malonic acid does not inhibit respiration of barley roots at about pH 5.6 and slightly stimulates it at pH 6.0, and Henderson and Stauffer also reported evidence of increased respiration of tomato roots in low concentrations of malonic acid. The writer has found no previous mention of the effect of pH on the inhibitory action of fluoride, but its activity seems to be affected as much by pH as is that of other inhibitors. BONNER and THIMANN (1) found that fluoride did not strongly inhibit the growth of pea coleoptiles at pH 6.0, but did not study its effect at other pH values. Laties attributes the greater effectiveness of inhibitors at lower pH values to their greater penetration into the tissues. Perhaps at the higher pH value so little malonic acid and fluoride penetrated the pine roots that they stimulated metabolic activity and consequently increased the accumulation of P^{32} instead of depressing it, but there is no proof of this hypothesis.

RETENTION OF P^{32} ON ROOT SURFACES

In connection with these studies questions arose concerning the amount of P^{32} adsorbed on the surfaces of the root segments by purely physical forces as compared to the amount accumulated within the cells of the roots. To study this, groups of 20 root tips and 20 mycorrhizal segments were selected. One-half of each group was killed by immersion in boiling water, and both the living and the dead roots were then immersed in solutions containing P^{32} for three hours. They were removed and washed in the usual manner, after which half of the living and half of the dead roots were immersed in distilled water for two and one-half hours and all of the roots were then dried and their activity determined. The results are summarized in table III. Although some phosphorus was held on the dead root tips, even after soaking for two and one-half hours, the quantity was negligible compared to the total amount retained by the living roots. A considerably larger quantity was held by the dead mycorrhizal roots, possibly because the fungal hyphae provide more surface on which adsorption can occur, and little of this was lost by soaking in distilled water. On the whole it appears that the amount of phosphorus held by adsorption on the surface of the

TABLE III

RETENTION OF P^{32} ON THE SURFACE BY LIVING AND DEAD PINE ROOTS. EACH VALUE IS AN AVERAGE OF THE COUNTS PER MINUTE FOR FIVE SEGMENTS.

Treatment	Root tips	Mycorrhizal segments
Living roots	26,611	4,728
Living roots soaked	21,920	2,957
Killed roots	903	665
Killed roots soaked	485	598

types of roots used in these experiments is not large enough to constitute a serious source of error.

Discussion

Most important in terms of the original objectives of this study are the relative effects of the inhibitors on accumulation of P^{32} by mycorrhizal and by nonmycorrhizal roots. The experiments were performed at various seasons of the year, using material from the forest as well as from pot-grown seedlings. Large differences occurred in absolute amounts of P^{32} accumulated by different samples, largely because of differences in volume and surface of different samples of root tips and mycorrhizal segments, and possibly because of differences in accumulative capacity of the tissue contained in different samples. Nevertheless, in all experiments with azide the mycorrhizal segments accumulated two or three times as much P^{32} as the root tips. At pH 4.7–4.8 fluoride also inhibited accumulation of P^{32} to a greater extent in nonmycorrhizal than in mycorrhizal roots, but at pH 5.7–5.8 it caused increased accumulation in both types of roots, although to a greater extent in mycorrhizal roots. Malonic acid did not significantly inhibit accumulation of P^{32} in either type of root at pH 4.7–4.8, but increased it above the control rate at pH 5.7–5.8, the increase being greater in nonmycorrhizal roots.

These results are in only partial agreement with those reported for roots of other species. MACHLIS (7) found that malonic acid at pH 5.0 and in the concentration used by the writer reduced intake of bromide by barley roots to less than half of his control rate. His results with azide were similar to those of the writer, complete inhibition of salt intake occurring. LATIES (6) found that 0.001 M fluoride at pH 5.0 reduced respiration of barley roots to about 20% of the control rate and 0.01 M malonate at pH 4.6–4.7 reduced respiration of barley roots to about 50% of the control rate. In our experiments malonic acid did not significantly reduce P^{32} accumulation at a low pH, and at pH 5.7–5.8 it caused an increase in accumulation. Fluoride almost completely inhibited it at a low pH and increased it at pH 5.7–5.8. HENDERSON and STAUFFER (2) found that neither fluoride nor malonate had much inhibitory effect on respiration of tomato roots at pH 5.2–5.8, but a low concentration of malonate appeared to increase respiration somewhat.

Although salt accumulation is not directly proportional to respiration, any treatment which seriously reduces respiration might be expected to reduce salt accumulation. The two processes would not necessarily be reduced to the same extent, however, as Machlis found that a concentration of azide and cyanide which completely stopped bromide accumulation by barley roots only inhibited about two-thirds of respiration, and low concentrations slightly increased respiration, but not bromide intake. Simultaneous measurements of respiration and of P^{32} accumulation would have been desirable, but lack of time made this impossible.

It seems clear that accumulation of P^{32} by nonmycorrhizal roots is reduced to a greater extent by azide, and at a low pH by fluoride, than is accumulation by mycorrhizal roots. Since these inhibitors presumably act through their effects on respiratory enzymes, our results suggest that there are differences in the enzyme systems of mycorrhizal and nonmycorrhizal roots. Azide is supposed to inhibit cytochrome oxidase, while fluoride, according to authors cited by Laties, is said to inhibit enolase and phosphatase. Malonic acid is said to inhibit succinic dehydrogenase. On this basis it might be suggested that in pine roots, as in barley and tomato roots, an enzyme system sensitive to azide plays an important part in respiration, but that it is more important in nonmycorrhizal than in mycorrhizal roots. The same is true with respect to fluoride, at least at pH 4.6, but neither type of root contains an essential enzyme system which is very sensitive to malonic acid. It would be very interesting to study separately the respiratory enzyme systems of nonmycorrhizal pine roots, of mycorrhizal roots, and of the fungus responsible for the development of mycorrhizae.

Summary

A study was made of the effects of various respiration inhibitors on the accumulation of P^{32} by mycorrhizal and nonmycorrhizal roots of loblolly pine. The inhibitors tested were 0.001 M sodium azide, 0.005 M sodium fluoride, and 0.025 M malonic acid. The roots were exposed to the inhibitors for an initial period of one hour, then exposed to inhibitor plus P^{32} having an activity of about 300 microcuries per liter for three hours.

Azide reduced accumulation to a greater extent in nonmycorrhizal than in mycorrhizal roots at all pH values. The inhibition was almost complete at pH 4.7, but much less at pH 5.7. Fluoride also almost completely inhibited accumulation at the lower pH, but increased it above the control rate at the higher pH. Malonic acid did not significantly reduce accumulation at the lower pH, but increased it above control at the higher pH. The pH of the system has a marked effect on the action of all three inhibitors.

In general pine roots seem to react to respiration inhibitors in about the same manner as barley and tomato roots, although barley roots appear to be more sensitive to malonic acid than pine roots.

It was concluded that, although both types of pine roots contain an azide- and a fluoride-sensitive respiratory enzyme system, that of non-

mycorrhizal roots is much more sensitive than that of mycorrhizal roots. It appears that the two types of roots are somewhat different physiologically.

Part of the work reported in this paper was aided by a grant from the Atomic Energy Commission. The author wishes to acknowledge the assistance of Dr. Karl M. Wilbur in the early part of this study and the cooperation of Dr. Philip Handler of the School of Medicine in providing certain facilities.

DEPARTMENT OF BOTANY
DUKE UNIVERSITY
DURHAM, NORTH CAROLINA

LITERATURE CITED

1. BONNER, W. D., JR. and THIMANN, K. V. Studies on the growth and inhibition of isolated plant parts. III. The action of some inhibitors concerned with pyruvate metabolism. Amer. Jour. Bot. **37**: 66–75. 1950.
2. HENDERSON, J. H. M. and STAUFFER, J. F. The influence of some respiratory inhibitors and intermediates on growth and respiration of excised tomato roots. Amer. Jour. Bot. **31**: 528–535. 1944.
3. HOAGLAND, D. R. The inorganic nutrition of plants. Chronica Botanica Co., Waltham, Massachusetts. 1944.
4. KELLY, S. The relationship between respiration and water uptake in the oat coleoptile. Amer. Jour. Bot. **34**: 521–526. 1947.
5. KRAMER, P. J. and WILBUR, K. M. Absorption of radioactive phosphorus by mycorrhizal roots of pine. Science **110**: 8–9. 1949.
6. LATIES, G. G. The role of pyruvate in the aerobic respiration of barley roots. Arch. Biochem. **20**: 284–299. 1949.
7. MACHLIS, L. The influence of some respiratory inhibitors and intermediates on respiration and salt accumulation by excised barley roots. Amer. Jour. Bot. **31**: 183–192. 1944.
8. STENLID, G. The effect of sodium azide on the exudation and oxygen consumption of excised plant roots. Physiologia Plantarum **1**: 185–195. 1948.