

THE EFFECT OF CERTAIN ENVIRONMENTAL CONDITIONS ON THE RATE OF DESTRUCTION OF VANILLIN BY A SOIL BACTERIUM

WILLIAM J. ROBBINS AND A. B. MASSEY

*Formerly Professor of Botany and Assistant Professor of Botany, respectively, at the
Alabama Polytechnic Institute and Experiment Station*

Received for publication July 15, 1920

One of the theories to explain why soils are or become infertile is the soil toxin theory which assumes that the failure of crops to grow on a soil is due to the presence of organic material in the soil which is injurious to the plants. Organic compounds exceedingly injurious to plants have been isolated from the soil. Among these compounds are the aldehydes, vanillin (10) and salicylic aldehyde (9). It has been shown that in at least some Alabama soils, the addition of vanillin has almost no injurious effects on plants (3) because it is rapidly destroyed by soil bacteria (4). It has also been shown that this is true of other soils to which the addition of vanillin has little or no effect on the growth of plants (6). It is true of some soils, however, that although the vanillin-destroying bacteria are present, added vanillin will persist and evidence its injurious effects on the growth of plants for a considerable period of time (6, 11). This persistence must be due to conditions which prevent the bacteria from acting on vanillin. In the present paper a study is made in solution cultures of the effect of certain conditions such as acidity, alkalinity, aeration and mineral salts on the decomposition of vanillin. The experimental work was performed in 1916 and the results, though fragmentary, are presented because conditions have prevented further investigation by the writers.

In order to study this question the vanillin-destroying bacterium isolated from Alabama soil was grown in synthetic culture solutions. What appears to be the same organism has also been isolated from Nebraska, New York and Virginia soils.

No complete study of this organism has been made. It is characterized by its ability to destroy vanillin and by its growth on beef-extract agar. On beef-extract agar, plus 1, it produces a cheesy yellow growth, wrinkled on the surface, and stains the medium yellow. It is aerobic, liquifies gelatine, grows moderately on potato and develops in peptone solutions containing dextrose, lactose, saccharose or glycerine without gas formation. It clears milk without coagulation.

No attempt has been made to determine how many species of bacteria there are which destroy vanillin. Two species have been isolated from the

soil, the one already referred to above and a second which produces no pigment on beef-extract agar. It is believed, however, that the ability to destroy vanillin is not a common property of soil bacteria and that the number of species which have the ability is limited.

DETERMINATION OF VANILLIN

Early in the study of the action on vanillin of the vanillin-destroying bacterium described above, it was found that the determination of the amount of vanillin by the method used was unreliable. The method first used was that described by Folin and Denis (2) with clarification by the lead acetate omitted. This method requires a reagent composed of phosphoric acid, sodium tungstate and phospho-molybdic acid and is referred to by them as the phenol reagent. On the addition of a saturated sodium carbonate solution, it produces with vanillin a deep blue solution, the depth of the color varying directly with the amount of vanillin present. Using the phenol reagent to determine the amount of vanillin in a synthetic nutrient solution in which the vanillin-destroying bacterium had grown, it was sometimes found that apparently more vanillin was present after the action of the bacterium than had been originally added. In some cases, twice as much vanillin was apparently formed in the inoculated culture as was present in an uninoculated, or check culture.

In an attempt to clear up this contradiction, the acid nitrate of mercury described by Estes (1) was used for determining vanillin. This reagent produces with vanillin a pink color, the depth of color varying directly with the amount of vanillin present. It was found that, determined by this means, the amount of vanillin progressively decreased in a synthetic nutrient solution containing vanillin and inoculated with a pure culture of the vanillin-destroying bacteria.

On further investigation, it was found that the anomalous results secured by the use of the phenol reagent were due to the fact that the bacterium oxidizes vanillin to vanillic acid (5) which produces a blue color with the phenol reagent 1.5–2.0 times as strong as is produced by an equal amount of vanillin. The depth of color yielded by a mixture of vanillin and vanillic acid with the phenol reagent, therefore, is the result of the color produced by both the vanillin and vanillic acid. The color production of vanillic acid with the Estes reagent however, is very slight so that vanillin can be determined in a mixture of vanillin and vanillic acid by the use of that reagent. Acid nitrate of mercury was therefore used for the determination of vanillin in the experiments reported below.¹

¹ It is probable that, by the use of lead acetate, a solution containing both vanillin and vanillic acid could be freed of the vanillic acid as it is precipitated by lead acetate in the presence of ammonia. The phenol reagent could then be used for the determination of the vanillin.

EFFECT OF ACIDITY AND ALKALINITY ON THE DECOMPOSITION OF VANILLIN

In determining the effect of acidity and alkalinity on the destruction of vanillin, the following nutrient solution was used:

K ₂ SO ₄	0.0340 gm.
NaNO ₃	0.1000 gm.
CaH ₄ (PO ₄) ₂	0.0710 gm.
Distilled Water.....	3000 cc.
Vanillin.....	As indicated

The solution was neutralized to phenolphthalein with NaOH, and aliquot parts made acid and alkaline with HCl and NaOH as noted in table 1. Forty cubic centimeters of solution were placed in 150-cc. Erlenmeyer flasks and after sterilization, 10 cc. of a sterile vanillin solution were added to each flask.² The organisms were allowed to grow 4 days at room temperature when the vanillin was determined by means of the acid nitrate of mercury reagent. Previous to the determination of the vanillin, the contents of each flask were neutralized.

TABLE 1

Effect of HCl and NaOH on the decomposition of vanillin

ADDITIONS PER 100 CC. OF SOLUTION	VANILLIN REMAINING IN CULTURE SOLUTION AT END OF 4 DAYS
	<i>p.p.m.</i>
10 cc. 0.1 N NaOH.....	260.0
5 cc. 0.1 N NaOH.....	41.6
2 cc. 0.1 N NaOH.....	24.0
1 cc. 0.1 N NaOH.....	23.6
Neutral to phenolphthalein.....	28.0
1 cc. 0.1 N HCl.....	274.0
2 cc. 0.1 N HCl.....	226.0
Checks, uninoculated.....	241.6

In this experiment which is the average of duplicate cultures, 1 cc. of 0.1 N HCl per 100 cc. was sufficient to inhibit the growth and action of the bacterium on vanillin. Between 5 and 10 cc. of 0.1 N NaOH were required, however, to stop its action. In this connection, it should be noted that Skinner and Noll (12) report that on unproductive soil the toxic effects of vanillin are overcome by liming. Truog and Sykora (13) also found that in an infertile acid sand, the poisonous action of the vanillin was greatly lessened by the addition of lime. A study of the relation of hydrogen-ion concentration to the decomposition of vanillin in solution cultures and sand should be made.

² This method of adding vanillin in solution after sterilization was used throughout when quantitative work was done, as it eliminated any danger of lack of uniformity in vanillin content of the flasks due to sterilization.

EFFECT OF AERATION ON THE RATE OF DECOMPOSITION OF VANILLIN

While studying the formation of the decomposition products of vanillin, it was observed that in a 2-liter Erlenmeyer flask containing 250 cc. of nutrient solution, the vanillin as tested for by the acid nitrate of mercury would disappear in three or four days. In the same flask, however, containing 1000 cc. of solution, vanillin was present after 3 weeks. The depth of solution in this case was about 7 cm. This difference was thought to be due to aeration. It should be noted, however, that the flask in which the slower digestion occurred had four times as much vanillin in it though the concentrations were the same.

To determine more definitely the effect of the depth of the solution on the rate of the disappearance of vanillin under the action of the vanillin-destroying bacterium, experiments were conducted in which 50 cc. of nutrient solution, made -0.2 Fuller's scale with NaOH, were placed in 2.5 by 25-cm. test-tubes and 150-cc. Erlenmeyer flasks. The quantity and composition of

TABLE 2

Effect of aeration on the rate of digestion of vanillin

Depth of solution in flask about 2 cm., in tube about 12 to 14 cm.

TIME FROM INOCULATION	VANILLIN IN FLASK		VANILLIN IN TUBE	
	Check not inoculated	Inoculated	Check not inoculated	Inoculated
<i>days</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>
3	242	108	226	190
5	210	24	220	78
7	306	8	292	40
9	234	0	224	34
11			246	14

the solution in both tubes and flasks were identical but in the tubes the depth of solution was 12 to 14 cm. with a diameter at the surface of the liquid of 2.5 cm. while in the flask the depth was about 2.0 cm. and the diameter at the surface of the liquid was 6.5 cm.

It is evident from the data in table 2 that the depth of the solution and the area of liquid surface exposed affect the rate of the decomposition of vanillin by this bacterium. The more rapid decomposition occurs in the flask where the depth of the solution is one-sixth of that in the tube and the liquid surface exposed is $2\frac{1}{2}$ times as great. This difference is no doubt due to the freer supply of oxygen offered in the case of the flask and is what would be expected when the destruction of vanillin by the organism is known to be an oxidative process.

THE EFFECT OF FERTILIZER SALTS ON THE DECOMPOSITION OF VANILLIN

It has been shown by Schreiner and Skinner (8) that fertilizer salts affect the toxicity of vanillin to wheat. The effects of calcium acid phosphate, sodium nitrate and potassium sulfate singly and in many combinations on

the toxicity of vanillin were studied by these investigators. The growth of wheat plants in culture solutions respectively high in phosphate, nitrate or potash and containing vanillin, showed that the vanillin depressed the growth least in the cultures high in nitrate and most in the cultures high in potash.

The writers have attempted to discover whether the same fertilizer salts have any effect on the rate at which vanillin is decomposed by the bacterium used in this investigation. The triangular diagram as used in physical chemistry was employed and the results are presented by its means. Monobasic calcium acid phosphate, sodium nitrate and potassium sulfate were used in 15 different combinations and in such concentrations that each culture solution contained a total concentration of 80 parts per million of P_2O_5 , NH_3 and K_2O . The mineral combinations used in parts per million of K_2O , P_2O_5 and NH_3 were as follows:

	K_2O	P_2O_5	NH_3
1	0	80	0
2	0	60	20
3	0	40	40
4	0	20	60
5	0	0	80
6	20	60	0
7	20	40	20
8	20	20	40
9	20	0	60
10	40	40	0
11	40	20	20
12	40	0	40
13	60	20	0
14	60	0	20
15	80	0	0

In preparing the culture solution sufficient of each of the three chemically pure salts was dissolved in distilled water to produce solutions containing 100 parts per million of P_2O_5 and K_2O , respectively.

This required:

0.1776 gm. of $CaH_4(PO_4)_2 \cdot H_2O$ per liter
 0.5000 gm. of $NaNO_3$ per liter
 0.1852 gm. of K_2SO_4 per liter

To prepare solution no. 1, 40 cc. of the $CaH_4(PO_4)_2$ were placed in a 150-cc. Erlenmeyer flask and after sterilization 10 cc. of a sterile vanillin solution were added, making a total of 50 cc. To prepare solution no. 7, 10 cc. of the K_2SO_4 , 20 cc. of the $CaH_4(PO_4)_2$ and 10 cc. of the $NaNO_3$ stock solution were placed in a 150-cc. Erlenmeyer flask and after sterilization, 10 cc. of a sterile vanillin solution were added.

Three separate experiments were performed. In each experiment the combinations of $CaH_4(PO_4)_2$, K_2SO_4 and $NaNO_3$ noted above were used. The

concentration of vanillin was uniform throughout a series. In experiment 1 (see table 3), 150-cc. Erlenmeyer flasks containing 50 cc. of solution were used and the concentration of vanillin was 508 parts per million. In experiment 2, the same containers and amount of solution were used but the experiment was performed in duplicate and the concentration of vanillin was 296 parts per million. In experiment 3, 50 cc. of solution were used in test-tubes approximately 2.5 by 25 cm. and the concentration of vanillin was 260 parts per million. Growth occurred at room temperature, the time varying with the experiment from 3 to 5 days. At the close of the experiment, the residual vanillin was determined by means of the acid nitrate of mercury reagent.

TABLE 3
Effect of fertilizer salts on the rate of decomposition of vanillin

SOLUTION	VANILLIN REMAINING IN CULTURES			
	Experiment I	Experiment II	Experiment III	Average
	p.p.m.	p.p.m.	p.p.m.	p.p.m.
1	48.0	148.0	37.0	77.6
2	18.8	108.0	60.0	62.3
3	16.8	102.0	100.0	72.9
4	32.0	114.0	96.0	80.6
5	19.2	180.0	128.0	109.1
6	56.4	110.0		83.2
7	40.4	116.0	62.0	72.8
8	167.6	118.0	118.0	134.5
9	164.0	152.0	80.0	132.0
10	532.0*	106.0	188.0	147.0
11	Trace	130.0	254.0*	65.0
12	176.0	160.0	228.0	188.0
13	80.0	146.0	36.0	87.3
14	54.0	242.0	12.0	102.7
15	160.8	146.0	156.0	154.3
Checks	508.0	296.0	260.0	354.7

* No decomposition. Omitted from averages.

The results indicate that the decomposition of vanillin proceeds most rapidly in those combinations of salts high in $\text{CaH}_4(\text{PO}_4)_2$. The complete data are given in table 3 where the amount of vanillin in parts per million remaining in each culture solution at the end of the experiment is given.

Using the averages for the three experiments given in column 4 of table 3, the total of the amounts of vanillin remaining in all those combinations of salts containing 50 per cent of $\text{CaH}_4(\text{PO}_4)_2$ or more, is 515.8 parts per million; in all those combinations of salts containing 50 per cent of NaNO_3 or more, is 717.1 parts per million; and in all those combinations containing 50 per cent of K_2SO_4 or more, is 744.3 parts per million.

It is evident that the decomposition of vanillin in these experiments proceeds most rapidly in the cultures high in $\text{CaH}_4(\text{PO}_4)_2$ and least rapidly in

the cultures high in K_2SO_4 . This fact is shown graphically in figure 1. The 15 cultures are here arranged on the triangular diagram. Each circle on the diagram represents a culture, the upper numbers indicating the solution and the lower numbers the amount of vanillin remaining in the solution after the action of the bacteria. The solutions at the corners of the diagram contain one salt only; cultures 1, 5 and 15 containing $Ca_2H_4(PO_4)_2$; $NaNO_3$ and K_2SO_4 , respectively. The cultures on the sides of the diagram contain combinations of two of

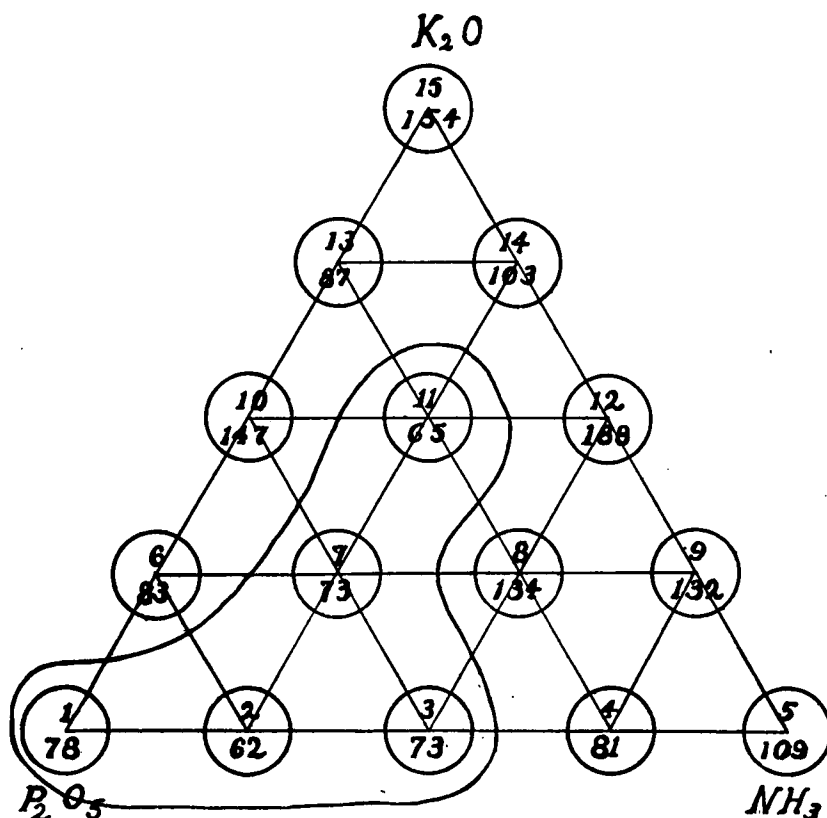


FIG. 1. TRIANGULAR DIAGRAM SHOWING THE EFFECT OF FERTILIZER SALTS ON THE RATE OF DECOMPOSITION OF VANILLIN

the salts and the cultures within the diagram contain combinations of the three salts in the proportions indicated earlier. An examination of the amounts of residual vanillin in the cultures shows that the smallest amounts are located in the corner of the triangle where the concentration of phosphate is highest. This is indicated in the figure by surrounding all those cultures containing less than 80 parts per million of residual vanillin by a line.

Although the effect of $Ca_2H_4(PO_4)_2$ on the rate of decomposition of vanillin is marked, it is impossible at the present stage of the investigation to state

the cause of its effect. Several explanations are possible. It may be that the balancing effect of the calcium on the other salts in the solution permits a greater growth of the bacteria and therefore greater digestion of vanillin in those solutions high in calcium. It may be a specific effect of the phosphate radical. The decomposition of vanillin by this organism is an oxidative process. Schreiner and Sullivan (7) have shown that phosphates and nitrates increase the oxidative power of roots. In this connection it is interesting to note that the same investigators in studying the effect of the addition of sodium nitrate, potassium sulfate and calcium acid phosphate to the soil on the oxidative power of the soil, found that of the three salts acid calcium phosphate produced the greatest increase in oxidation in four soils out of five (table 15) and was greatest or second greatest in effect in all but one trial with a second lot of four soils (table 14).

EFFECT OF GLUCOSE ON THE DECOMPOSITION OF VANILLIN

To determine whether the presence of an easily fermentable carbohydrate such as glucose would affect the action of the bacterium on vanillin, experiments were conducted in synthetic nutrient cultures to which both vanillin and glucose had been added. It was found that the vanillin and glucose were used by the bacterium simultaneously and that the effect of the glucose on the destruction of vanillin was not marked.

DISCUSSION

In the work of which the present paper is the fourth report, we have tried to determine why organic toxic compounds were injurious to plants when added to some soils but not injurious when added to others. Previous investigators have assigned the more important role in the amelioration of the toxic effect of organic compounds to the physical action of the soil, to the chemical reactions which take place in the soil between the toxins and soil constituents or to the oxidative and reducing ability of plant roots. We have found that by far the most important role in the amelioration of the toxicity of organic compounds in the soil is played by the destructive action of soil microorganisms; in some cases by the action of specific bacteria. It is a well recognized fact that the ability to destroy some organic compounds is not a common property of all bacteria but for many organic compounds there are a limited number of species which have the power of destroying them. The complete disintegration of a mass of dead plants or animals is not accomplished by one species of microorganisms but is accomplished by the successive action of many. One group acts on the end-products of another. If at any stage the suitable organisms are not present or conditions are such that they cannot act, then the material which they usually break down will accumulate. This accumulation may be only temporary, disappearing with a change in conditions, or it may be more permanent, as in humus formation and the

formation of peat. But in any case, the destruction or accumulation is basically a function of the action of microorganisms.

While it is impossible to apply too strictly to the soil the results from solution cultures, we have shown in the present paper that alkalinity and good aeration favor the destruction of vanillin, because of their effect on the action of the vanillin-destroying bacteria on vanillin. We have also shown that some of the salts commonly used as fertilizer elements affect the rate at which vanillin is destroyed by bacteria. If it can be shown that the opposite conditions, acidity, poor aeration and the abundance or deficiency of some mineral salts allow the formation of vanillin to proceed, then the conditions for its accumulation and action as a factor in soil fertility are defined.

SUMMARY

1. It is believed that the number of species of soil bacteria able to destroy vanillin is limited.
2. Slight concentrations of HCl inhibit the action on vanillin of the soil bacterium studied.
3. Aeration favors the destruction of vanillin by the organism used.
4. In solution cultures containing $\text{CaH}_4(\text{PO}_4)_2$, NaNO_3 and K_2SO_4 singly or in combination and inoculated with the bacterium used, vanillin is destroyed most rapidly in those solutions high in $\text{CaH}_4(\text{PO}_4)_2$ and least rapidly in the solutions high in K_2SO_4 .
5. The presence of glucose has no marked effect on the rate at which vanillin is destroyed by the bacterium used.

REFERENCES

- (1) ESTES, C. 1917 A new qualitative test and colorimetric method for the estimation of vanillin. *In* Jour. Indus. Engin. Chem., v. 9, no. 2, p. 142-144.
- (2) FOLIN, O., AND DENIS, W. 1912 A new colorimetric method for the determination of vanillin in flavoring extracts. *In* Jour. Indus. Engin. Chem., v. 4, no. 9, p. 670-672.
- (3) FUNCHES, M. J. 1916 The effects of certain organic compounds on plant growth. Ala. Agr. Exp. Sta. Bul. 191, p. 103-132.
- (4) ROBBINS, W. J. 1917 The cause of the disappearance of coumarin, vanillin, pyridine and quinoline in the soil. Ala. Agr. Exp. Sta. Bul. 195, p. 49-64.
- (5) ROBBINS, W. J., AND LATHROP, E. C. 1919 The oxidation of vanillin to vanillic acid by certain soil bacteria. *In* Soil Sci., v. 7, p. 475-485.
- (6) ROBBINS, W. J., assisted by A. E. ELIZANDO. 1918 The destruction of vanillin in the soil by the action of soil bacteria. Ala. Agr. Exp. Sta. Bul. 204, p. 125-131.
- (7) SCHREINER, O., AND SULLIVAN, M. X. 1910 Studies in soil oxidation. U. S. Dept. Agr. Bur. Soils Bul. 73, p. 1-57.
- (8) SCHREINER, O., AND SKINNER, J. J. 1911 Organic compounds and fertilizer action. U. S. Dept. Agr. Bur. Soils Bul. 77.
- (9) SHOREY, E. C. 1913 Some organic soil constituents. U. S. Dept. Agr. Bur. Soils Bul. 88.

- (10) SHOREY, E. C. 1914 The presence of some benzene derivatives in soils. *In Jour. Agr. Res.*, v. 1, no. 5, p. 357-363.
- (11) SKINNER, J. J. 1915 Field tests with a toxic soil constituent, vanillin. U. S. Dept. Agr. Bul. 164.
- (12) SKINNER, J. J., AND NOLL, C. F. 1916 Field tests of fertilizer action on soil aldehydes. *In Jour. Amer. Soc. Agron.*, v. 8, p. 273-298.
- (13) TRUOG, E., AND SYKORA, J. 1917 Soil constituents which inhibit the action of plant toxins. *In Soil Sci.*, v. 3, no. 4, p. 333-351.