FRENCHING SYMPTOMS PRODUCED IN *NICOTIANA TABACUM* AND *NICOTIANA RUSTICA* WITH OPTICAL ISOMERS OF ISOLEUCINE AND LEUCINE AND WITH *BACILLUS CEREUS* TOXIN

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(WITH TWO FIGURES)

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It has been suggested by the writer that the stages leading to abnormal growth in frenching of field tobacco may be as follows: bacterial soil toxin → receptor → isoleucine → frenching. The chemical composition of the bacterial soil toxin is unknown as yet. However, natural leucine as well as isoleucine was found to be capable of producing these growth abnormalities in *Nicotiana rustica*. Natural isoleucine can produce the symptoms of frenching in tobacco (**1, 3**) and in *N. rustica* (**5**). The natural isomer of leucine is also effective with *N. rustica* (**5**). The actions of other optical isomers of isoleucine and leucine are unknown.

Frenching of tobacco in the laboratory has been produced with a common soil microörganism, *Bacillus cereus* Fr. and Fr. (**2, 4**). Though this bacillus is not known to invade the tissues of the tobacco plant, rhizosphere and rhizoplane counts increase by 65 and 200%, respectively, when frenching occurs in the field (**6**). Diffusates of *B. cereus* in aseptic culture appear to be capable of exercising a similar action (**4**). The absence of free amino acids in soil and the marked increase of free isoleucine in affected leaves led to the assumption that the bacterial toxin and isoleucine were probably not identical (**7**). No free amino acids could be detected in soil by the Van Slyke method for determining free amino acids which is sensitive to 0.01% or 100 p.p.m., the approximate concentration at which isoleucine would have to be present to be effective.

Data in this paper cover the frenching responses of tobacco treated with optically active isomers of isoleucine, leucine, and two dipeptides of leucine. The effects of various impure preparations of bacterial toxin from *B. cereus* were also studied.

Methods

The tests upon seedlings of the action of organic compounds and of bacterial toxin were made necessarily in aseptic culture. The methods used have been published in previous papers (**1, 2**). Briefly, they consisted in growing tobacco seedlings in aseptic culture on mineral agar from sterilized seed. Either 200 ml. or 1000 ml. flasks were used. A light intensity of about 500 foot-candles was furnished by white fluorescent lamps. Usually, the experiments with *N. tabacum* were carried out in 200 ml. flasks at 25° C, and those with *N. rustica* in 1000 ml. flasks at room temperature. The former were grown for four to six weeks, the latter, two to three months. All tests were in duplicate.

Two tobacco varieties were used in these experiments. One was Maryland Medium Broadleaf, the other was Havana 142, a Wisconsin cigar tobacco. The *N. rustica* variety was Mahorka 1 originating in Siberia (5).

D-Leucine, L-alloisoleucine, and D-alloisoleucine, were obtained through the courtesy of Dr. Jesse P. Greenstein of the U. S. Public Health Service; and γ-aminobutyric acid from Dr. F. C. Steward of Cornell University. The other amino acids were commercial preparations.

Results

Optical isomers

The data for *N. rustica* in table I were obtained with one liter flasks at room temperatures for a growth period of several months. It will be noted that only the natural isomers of isoleucine and leucine belonging to the l-family caused the seedlings to french. Leucine appeared to be more effective than isoleucine. Far more effective than either of these was L-alloiso-

TABLE I

EFFECTS OF THE OPTICAL ISOMERS OF ISOLEUCINE AND LEUCINE ON GROWTH OF *N. rustica* (MAHORKA 1).

Compound	p.p.m.	At budding			At flowering			Symptoms of toxicity
		Days	Leaves		Days	Leaves	Bracts	
				Cm.				
None	....	30	9.3	13.0	36.3	9	5.0	Normal
L(+)-Isoleucine	100	....	....	13.8*	....	....	....	Chlorotic, narrow leaves
	200	....	....	10.3*	....	....	....	Strap leaves
	300	....	....	10.0*	....	....	....	Strap leaves
D(−)-Isoleucine	100	29	9.5	11.8	35.5	8.5	5.0	Normal
	200	37.5	8.5	8.5	45.0	10.5	3.0	Small
DL-Isoleucine	100	82.5	15.0	15.0	82.0			Chlorotic, narrow leaves
	200	....	....	11.8*	....	....	....	Strap leaves
L(−)-Leucine	50	38.0	10.0	8.0	46.0	11.0	3.5	Chlorotic, narrow leaves
	100	51.5	15.8	8.3	60.8	17.5	2.5	Strap leaves
	200	45.0	19.0	10.0	53.0	22.0	0	Strap leaves
D(+)-Leucine	20	....	....	6.0*	....	....	....	Interveinal chlorosis
	50	94.0	15.0	6.0	102.0	15	0	Marginal chlorosis
	100	....	....	3.8*	....	....	....	Marginal chlorosis
L-Alloisoleucine	50	....	....	1.0*	....	....	....	Strap leaves
	100	....	....	1.0*	....	....	....	Strap leaves
	200	....	....	1.0*	....	....	....	Strap leaves
D-Alloisoleucine	200	40.0	8.5	6.0	47.5	9.0	5.0	Normal
L-Leucylglycine	200	45.5	10.5	13.5	47.5	11.5	3.0	Normal
Glycyl-L-Leucine	200	34.0	8.0	8.5	42.0	10.0	3.0	Normal
γ-Aminobutyric acid	50	33.0	8.0	11.5	38.0	8.0	5.0	Normal
	100	33.0	8.0	10.5	38.0	8.0	5.0	Normal

*Height at harvest.

TABLE II

EFFECTS AT 200 P.P.M. OF OPTICAL ISOMERS OF ISOLEUCINE AND LEUCINE ON GROWTH OF MARYLAND MEDIUM BROADLEAF TOBACCO.

Compound	Symptoms of toxicity	
	Time in days	Description
None	....	Normal
L-Alloisoleucine	6	First leaf formed was strapped
D-Alloisoleucine	....	Normal
D-Leucine	7	Retarded; pale; small round leaves, long petioles
L-Leucine	....	Normal
L-Isoleucine	13	Strap leaves
D-Isoleucine	....	Normal

leucine. D-Leucine, D-isoleucine, and D-alloisoleucine did not cause frenching responses to appear. Coupling of L-leucine with glycine to form a dipeptide destroyed its frenching properties. The tests with γ-aminobutyric acid are included because its action on growth of *N. rustica* seedlings had not been studied previously.

The action of the optical isomers of isoleucine and leucine on growth of Maryland Medium Broadleaf tobacco is illustrated in table II. Only L-isoleucine and L-alloisoleucine were effective in causing frenching symptoms. L-Alloisoleucine was more effective in causing growth abnormalities than was L-isoleucine. A growth period of four weeks at 25° C, was used in this experiment. The flasks were of 200 ml. capacity.

Frenching of tobacco ordinarily starts with a reticular chlorosis of the unexpanded upper leaves, and an inhibition in elongation of the main axis. If severe enough, the new leaves that develop are strapped, *i.e.*, consist almost entirely of midribs. A notation of strap-leaves indicates usually that the reticular chlorosis stage had occurred also. Where strap-leaves were formed after a very brief interval, the reticular chlorosis stage may be bypassed. The action of L-alloisoleucine in table II at 200 p.p.m. took place in such a manner.

A comparison of the relative effectiveness of isoleucine and alloisoleucine was made at 25° C with Maryland tobacco seedlings. Flasks of 200 ml.

TABLE III

RELATIVE EFFECTIVENESS OF L-ISOLEUCINE AND L-ALLOISOLEUCINE IN CAUSING THE FORMATION OF FRENCHING SYMPTOMS IN MARYLAND MEDIUM BROADLEAF TOBACCO.

Compound	Reticular chlorosis		Narrow leaf		Strap-leaves	
	Days	p.p.m.	Days	p.p.m.	Days	p.p.m.
L-Isoleucine	16	8	16	16	22	40
L-Alloisoleucine	19	2	12	4	12	8

capacity were used. It is evident on examination of table III that whether comparison is made at the reticular chlorosis stage, at formation of narrow leaves, or at strap-leaves, the results are the same. L-Alloisoleucine was about four times more effective than L-isoleucine in causing the formation of symptoms of frenching in tobacco seedlings.

Figure 1 illustrates the relative effectiveness of these two isomers at the 16 p.p.m. level. The seedlings were 41 days old. Addition of L-alloisoleu-

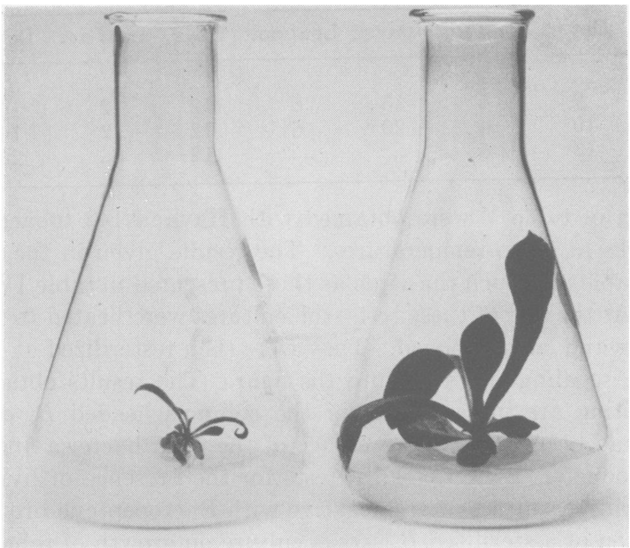

FIG. 1. Maryland Medium Broadleaf seedlings 41 days old. Seedling at left treated with L-alloisoleucine and that on right with L-isoleucine. Both amino acids were used at the 16 p.p.m. level.

cine resulted in greatly decreased growth of seedling which showed four strap-leaves. The seedling treated with L-isoleucine at 16 p.p.m. was much larger, and its leaves were perceptibly narrow but not strapped.

B. cereus TOXIN

Table IV is a repetition and extension of trials previously reported (**4**) for Maryland tobacco seedlings. With none of the methods of inoculation of the agar was there any evidence of bacterial spread. Bacterial growth was localized at the point of inoculation. The cup type of inoculation refers to the use of small sintered-glass Pyrex crucibles capable of retaining bacteria. Only the medium inside the crucible was inoculated. Nevertheless, the products of diffusion from the bacteria caused frenching. Transfers of the agar medium at a distance from the point of inoculation to Bactopeptone broth did not show the presence of viable bacteria. The experiments were carried out at 25° C for four weeks using 200 ml. flasks. Havana 142 is more sensitive to the action of isoleucine than is Maryland Medium Broadleaf tobacco.

TABLE IV

EFFECT OF TYPE OF AGAR INOCULATION (*B. cereus*) ON THE PRODUCTION OF FRENCHING SYMPTOMS IN ASEPTICALLY CULTURED TOBACCO SEEDLINGS.

Inoculation	Maryland Medium Broadleaf				Havana 142			
	Initial chlorosis		Initial strapping		Initial chlorosis		Initial strapping	
	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.
Control	....	....	....	....	....	....	....	....
Stab	9	4	15	6	12	2	16	5
Smear	10	4	20	6	12	2	16	5
Cup	13	6	....	....	12	....	28	7

The data of table V were obtained with Havana 142 tobacco grown in 200 ml. flasks at room temperatures. The results given in the table under *B. cereus* viable are much the same as those presented in table IV under Havana 142. At the end of these tests, the cultures were heated to 100° C, and the seedlings and cups removed. They were then resterilized at 120° C, and fresh sterile seedlings inserted into the agar. The results obtained on the second planting are indicated under the columns headed *B. cereus* dead. Frenching had occurred in the presence of the dead bacteria and its heated diffusion products. Bacteriological tests for the presence of living bacteria in the resterilized cultures were negative with Bactopeptone broth.

The action of a sterilized *B. cereus* culture on growth of tobacco is illustrated in figure 2. Chlorosis and extreme strapping of the leaves are evident. The appearance of the seedling is quite characteristic of frenching.

Discussion

The morphological abnormalities characteristic of frenching are: (a) the cessation of stem and branch growth, flowering also being inhibited or prevented; (b) reticular chlorosis of incompletely developed top leaves in the early stages of the disease; and (c) non-expansion of leaf lamina, *i.e.*, strap-

TABLE V

EFFECT OF HEAT ON THE CAPACITY OF *B. cereus* CULTURES TO CAUSE FRENCHING SYMPTOMS IN HAVANA 142 TOBACCO.

Inoculation	*B. cereus* viable				*B. cereus* dead			
	Initial chlorosis		Strap leaves		Initial chlorosis		Strap leaves	
	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.
Control	....	....	....	....	....	....	....	....
Stab	8	3	....	....	9	2	....	....
Smear	....	....	....	....	....	....	....	....
1 cup	7	3	22	7	9	2	13	3
2 cups	6	2	20	4	13	....	24	....

ping of immature leaves. These three hormone-like effects were limited to L-isomers of alloisoleucine, isoleucine, and leucine. Substitution in the amino or carboxyl groups of L-leucine destroyed its frenching activity. It is possible, therefore, that neither leucine nor isoleucine exercises a direct action in causing frenching symptoms, since leucine is ineffective with tobacco and more effective than L-isoleucine with *N. rustica.* Both possibly represent intermediate stages in causation of this response.

Whether L-alloisoleucine enters into these reactions is unknown. It is not known to occur naturally. Were it not for this, the simplest explanation with the facts on hand would be that L-alloisoleucine is the bacterial toxin causing frenching. Its effective minimal range for chlorosis and strapping

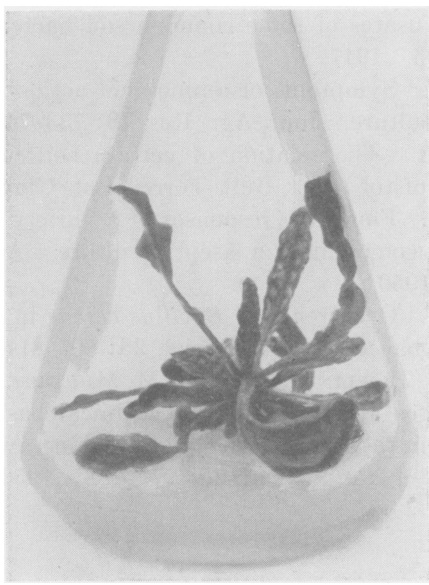

FIG. 2. Maryland Medium Broadleaf seedling grown in a heat-sterilized culture of *B. cereus* for 49 days and showing symptoms of frenching.

in agar cultures is 2 to 8 p.p.m. or less. In water culture, it should prove 5 to 10 times more effective, if it performs similarly to isoleucine. No definite conclusion can be reached, however, until the chemical structure of the bacterial toxin becomes known.

Summary

Nicotiana tabacum (Maryland Medium Broadleaf and Havana 142) and *N. rustica* (Mahorka 1) were grown aseptically on mineral agar under about 500 fc of white fluorescent light. Addition of either L-isoleucine or L-alloisoleucine produced symptoms of frenching in both species. L-Leucine was also effective with *N. rustica.* Relative effectiveness on tobacco was alloisoleucine > isoleucine; on *N. rustica,* alloisoleucine > leucine > isoleucine. L-Alloisoleucine was four times more effective (minimal range 2 to

8 p.p.m.) than L-isoleucine with tobacco. D-Isoleucine, D-leucine, and D-alloisoleucine did not cause frenching symptoms. Neither did glycyl-L-leucine nor L-leucyl-glycine. A heat stable toxin from *Bacillus cereus* Fr. and Fr. also caused production of frenching symptoms in tobacco.

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