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FURTHER STUDIES ON THE ACTION OF FREE AMINO ACIDS ON FLOWERING OF DUCKWEED, *LEMNA GIBBA* G3

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1. The maximum inhibition of flowering of a long-day duckweed, *L. gibba* G3, occurred when amino acid was added to the culture medium on the third long-day. Lysine could reverse the inhibition non-specifically.

2. When the concentration ratio of endogenous arginine to endogenous lysine was under 10, the flowering processes progressed normally, and the flower production decreased with the increase of the ratio from 10 to 20.

3. These and relevant findings support our previous conclusion that in vivo free amino acids may contribute in establishing intracellular state determining whether certain essential reaction(s) involved in flowering processes can proceed normally or not.

The amino acid given singly to higher plants is known to exert sometimes inhibition or modification on the development of the whole plant or parts of it (*1-3*). For example, in the presence of applied glycine, the seedling of *Oenanthe aquatica* produces "neomorph". With respect to the cellular compositions, this abnormal plant does not differ from normal one (grown in the absence of exogenous glycine), except that the former contains a remarkably higher amount of soluble nitrogen compounds. MIETTINEN and WARIS have suggested that glycine does not inhibit cell division but inhibits the differentiation in the plant in question (*4*).

In our previous report (*5*), it was shown that the flowering of a long-day duckweed, *Lemna gibba* G3, was inhibited by amino acid added singly, and the inhibition by arginine was reversed by lysine. The relative concentrations of endogenous amino acids were suggested to play some important role in the flowering processes. Furthermore, the maximum effects were brought forth when the amino acids were applied in the incipience of differentiation of granular body (cf. *6* for the definition of granular body) to flower primordium. In the present report, analytical results are presented which indicate that the concentration ratio of certain pairs of endogenous amino acids may control the flowering process, that there may be rather restricted number of effective pairs of amino acids, and that amino acids may not act as metabolic intermediates or antagonists but may contribute

to establish some in vivo state regulating essential reaction(s) involved in the flowering process.

MATERIAL AND METHODS

The experimental material was a long-day strain of duckweed, *L. gibba* G3. The methods of experimental culture and count of frond and flower numbers were the same as those reported previously (*5*). Short- and long-day regimes used were a combination of 10 hours of light and 14 hours of dark, and a continuous light, respectively. M-medium supplemented with 1% sucrose was used as the basal medium. All cultures were conducted aseptically at ca. 26°. The degree of flowering was expressed in terms of FL % (number of flowers/number of fronds) according to HILLMAN (*7*).

Ethanol soluble fraction was prepared as follows: After being washed five times with distilled water, fronds were extracted three times with 75% (v/v) ethanol at 80°. Residue was washed twice with 75% (v/v) ethanol at room temperature. All alcoholic extracts and washings were combined and concentrated in vacuo to remove alcohol. Aliquots of the concentrated extract were used for amino acid analyses. Total soluble amino acid was estimated by the method of ROSEN (*8*). Acidic, neutral and basic amino acid fractions were separated by means of paper electrophoresis (*9*). The spots stained with ninhydrine (*10*) were cut off and eluted with 60% ethanol to measure optical density at 570 mμ. The relative contents of acidic, neutral and basic amino acids were obtained by dividing optical density of respective fractions by the sum of optical density of the three fractions. Arginine and lysine were fractionated by Amberlite XE-64 column chromatography (*11*) and measured by the method of YEMM and COCKING (*12*). The recoveries of arginine and lysine were 100±5 and 100± 10%, respectively. Fractionated arginine and lysine were identified by elution pattern and co-chromatography with authentic samples (both column and paper chromatographies examined; effluent fractions of arginine and lysine were desalted with (H^+-type) Dowex-50 prior to paper chromatography). All experiments were repeated more than twice, and since each gave at least qualitatively similar results, only typical ones will be shown below.

EXPERIMENTAL RESULTS

1. *Effect of amino acids added at different times of culture period*

Arginine inhibits flowering most effectively when applied on the 3 day of culture under continuous light (*5*). The same was found to be also true for other inhibitory amino acids. The fronds were exposed to a combination of 4 long-days and subsequent 4 short-days. Each amino acid was applied for 24 hours of the 3 or the 4 long-day, or throughout the 4 short-

TABLE I

Effect of amino acids added at different times on flower production in L. gibba *G3*

Amino acid	Concentration (mg/liter)	FL % (expressed as per cent of FL % of control)		
		3 day[a]	4 day[a]	5–8 days[a]
L-Glutamic acid	400	33	85	101
D, L-Tyrosine	10	0	38	58
L-Aspartic acid	600	24	38	79
D, L-Cysteine	100	0	33	94
D, L-Threonine	50	0	43	82
D, L-Methionine	10	3	45	42
L-Histidine	20	0	35	54
L-Hydroxyproline	1	17	54	43
D, L-Tryptophan	100	8	55	62
D, L-Serine	100	4	13	67
D, L-Phenylalanine	100	6	30	69
D, L-Cystine	10	26	60	—
L-Proline	100	2	30	72
Glycine	100	12	40	88
D, L-Alanine	17.8	19	46	100
L-Valine	7	8	23	59
L-Leucine	2	14	69	88
D, L-Isoleucine	1.5	16	63	69

[a] Time of application of amino acid.

Duckweed was exposed to 4 long-days and the numbers of fronds and flowers were counted after subsequent 4 short-days. See text for further experimental details.

days.

The inhibition of flowering by the amino acids tested was always maximum when they were added on the third long-day. This suggests that on the third long-day a reaction system(s) sensitive non-specifically to exogenous amino acids is operating.

2. Periodical change in sensitivity to amino acids

Fronds were cultured for 3 to 8 long-days combined with subsequent 4 short-days, the dark period of the first short-day being scheduled to come just after the end of the last long-day. Fronds were placed on arginine-containing medium for 24 hours of the last long-day, and, then, were returned to fresh medium after being well washed with arginine-free medium. When arginine was added on the third long-day, fronds were subjected to one more long-day, after they were transferred to arginine-free medium, to ensure flowering (cf. *5*, *6*). As seen in Fig. 1., the flowering response of the duckweed to arginine changed apparently periodically. Thus the sensitivity to the amino acid was lowest on the 5th long-day and was elevated considerably either before or after this

period. That in the initial 3 days the sensitivity to arginine may change as indicated by a broken line in Fig. 1 has been demonstrated previously (*5*). In view of the finding that other amino acids also evoke the greatest inhibition when they are added on the 3 long-day (Table I), the sensitivity of the duckweed to these amino acids may be expected to change in the same periodical way as suggested for arginine.

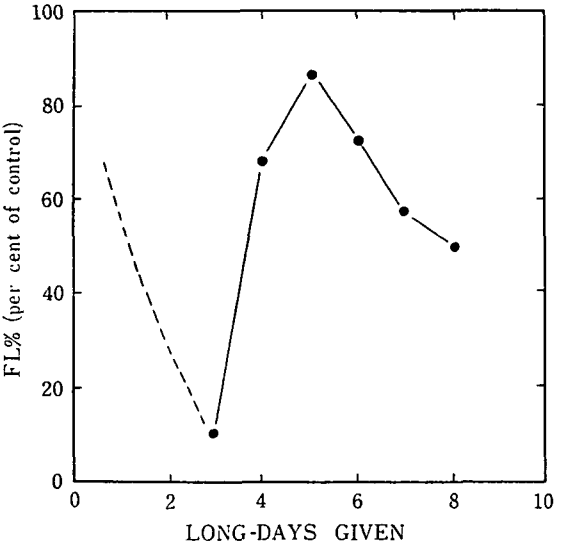


Fig. 1. Change in sensitivity to arginine under long-day condition. Duckweed was subjected to 4 to 8 long-days, and the numbers of fronds and flowers were counted after subsequent 4 short-days. L-Arginine (7.1×10^{-4} M) was added to culture medium for 24 hours of the last long-day. Exceptionally, when arginine was applied on the third long-day, one more long-day was given before subsequent short-days.

3. *Reversal by lysine of the floral inhibition by amino acids*

The inhibition of flowering by arginine has been reported to be reversed by lysine, and the reversal is maximum when the molar concentration ratio of exogenous arginine to exogenous lysine is 3 (*5*). Table II shows that lysine could also reverse the inhibition by nearly all other amino acids examined. If certain amino acids (e.g., lysine) can reverse non-specifically the floral inhibition caused by other amino acids, the species of such pairs of amino acids which may mutually antagonize will be rather restricted in number. And in each of such pairs as serine-lysine, arginine-threonine, arginine-cysteine and lysine-arginine, the latter partner was found to be unable to reverse the inhibition caused by the former. That some amino acids can alleviate non-specifically the inhibitory effect of other amino acids, as was the case for lysine, is quite similar to the HARRIS' finding (*1*) of

non-specific reversal by amino acids of plant development inhibited by other amino acids.

In the next experiment, fronds were cultured on arginine-containing medium for 4 long-days combined with subsequent 4 short-days. Lysine

TABLE II

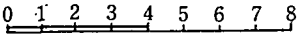
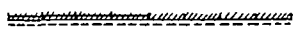
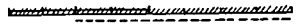
Reversal by lysine of the floral inhibition caused by other amino acids

Expt. No.	Amino acid	Concentration (mg/liter)	L-Lysine (30 mg/liter)	FL %	Frond number (per cent of control)
1	D, L-Serine	100	−	0	124
			+	0	108
	L-Proline	100	−	2	128
			+	16	101
	D, L-Threonine	50	−	0	119
			+	0	16
	D, L-Phenylalanine	100	−	5	130
			+	17	113
	Control (no addition)			28	100
2	D, L-Alanine	20	−	0	32
			+	32	106
	D, L-Methionine	20	−	0.3	149
			+	17	117
	L-Histidine	20	−	2	67
			+	23	83
	Glycine	100	−	4	117
			+	45	101
	Control (no addition)			39	100

L-Lysine (30 mg/liter) and other amino acids at designated concentrations were given for 8 long-days from the start of culture. At this concentration lysine alone did not affect the floral production. +; L-lysine added. −; L-lysine not added.

TABLE III

Reversal by lysine, applied at various times, of the floral inhibition by arginine

Culture schedule (day) 0 1 2 3 4 5 6 7 8	FL %	Frond number (per cent of control)
	13	100
	0.3	216
	12	156
	9	195
	1	216
	1	207

Duckweed was exposed to a combination of 4 long-days and subsequent 4 short-days. Oblique line, 7.1×10^{-4} M L-arginine applied; broken line, 2.2×10^{-4} M L-lysine applied; double line, long-day; single line, short-day.

was added at designated times to medium. The flowering responses at the end of the short-day treatment (Table III) indicate clearly that lysine was effective only when it was applied during the first 3 long-days. The result is not inconsistent with the fact that arginine inhibits flowering on the 3rd long-day.

4. Amino acid pattern of fronds induced and non-induced to flower

The experimental design together with the results obtained are illustrated in Fig. 2; total soluble amino acid was estimated at the end of 3 days of culture. As seen, little difference in the acid contents was detectable between the induced (Expt. Nos. *1*, *4*) and non-induced fronds (Expt. Nos. *2*, *3*, *5*).

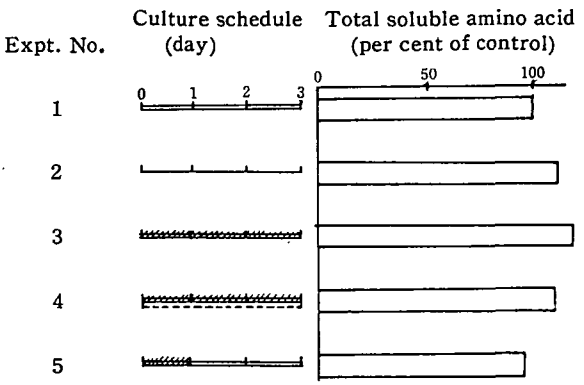


Fig. 2. Content of total soluble amino acid in induced and non-induced fronds. Fronds were analysed after 3 days of culture. See the explanation of Table III for symbols used. 7.1×10^{-4} M L-arginine and 2.2×10^{-4} M L-lysine applied.

Fig. 3 shows the ratios of acidic, neutral or basic soluble amino acid to total soluble amino acid determined for the same samples as used in the preceding experiment (Fig. 2).

Fronds fed with arginine contained larger amount of basic amino acid than those cultured on basal medium did. This alone, howevr, could not explain the inhibition of flowering by arginine, as a similar preponderance of basic acids was observed in the fronds cultured on medium containing both arginine and its antagonist, lysine. Table IV shows the contents of arginine and lysine in ethanol soluble fraction obtained from fronds cultured on basal medium for 3 long-days. In spite of fluctuation in the absolute amounts of both amino acids, the content ratio of arginine to lysine was found to be nearly constant at about 10.

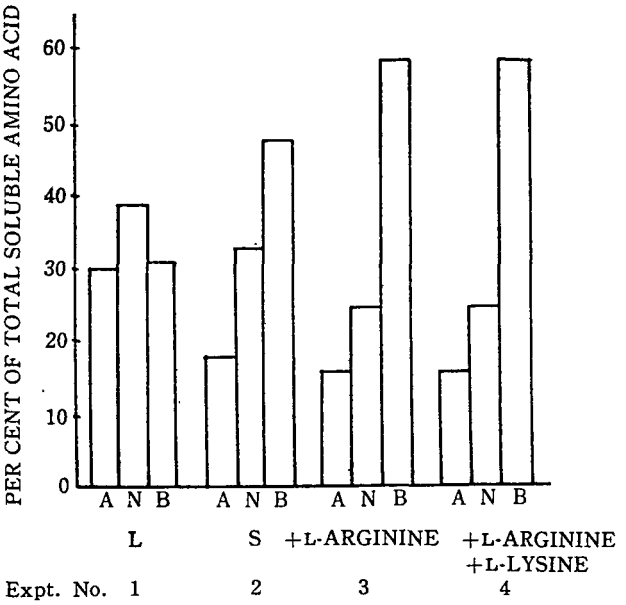


Fig. 3. Change in relative content of acidic, neutral and basic soluble amino acids with culture conditions. The same samples as used in the preceding experiment (Fig. 2) were analysed. Expt. Nos. correspond to those in Fig. 2. L, long-day; S, short-day; A, acidic amino acid; N, neutral amino acid; B, basic amino acid.

TABLE IV

Contents of arginine and lysine in ethanol soluble fraction obtained from induced duckweed

Expt. No.	Amino acid content (μmole leucine equiv./g F.W.)		Arginine/Lysine
	Arginine	Lysine	
1	1.2	0.11	11
2	2.1	0.19	11
3	3.1	0.37	8
4	2.2	0.24	9
5	2.4	0.23	10

Duckweed was analysed after it was cultured on basal medium for 3 long-days.

From the results shown in Tables IV and V, it may be suggested that the in vivo ratio of arginine to lysine has something to do with flowering process, which appears to progress normally only when the ratio is under 10.

TABLE V

Contents of arginine and lysine in ethanol soluble fraction obtained from induced and non-induced duckweed

Culture condition		Amino acid content (μmole leucine equiv./g F.W.)		Arginine/Lysine
		Arginine	Lysine	
	Long-day	2.1	0.22	10
	Short-day	3.5	0.26	13
Expt. 1	Long-day			
	+Arginine	7.5	0.28	27
	+Arginine+Lysine	7.6	0.72	11
	Short-day			
	9 am (just after dark period)	4.5	0.26	17
Expt. 2	7 pm (just before dark period)	3.8	0.30	13
	Long-day			
	9 am	2.2	0.24	9
	7 pm	2.4	0.23	10

Duckweed was cultured under designated conditions for 3 days. 7.1×10^{-4} M L-arginine and 2.2×10^{-4} M L-lysine were added at the start of culture. In Expt. 2, fronds were harvested at 9 am and 7 pm of the third day to be analysed.

The relationship between the ratio and the flower production was examined and the results obtained are shown in Table VI. Arginine did not inhibit flower production when the ratio was under 10, but caused inhibition in proportion to the increase of this ratio from 10 to 20. The

TABLE VI

Flower production as a function of the ratio of arginine to lysine

Amino acid added ($\times10^{-4}$ M)		Content ratio (in vivo): Arginine/Lysine	FL %
Arginine	Lysine		
0	0	7	19
7.1	0	19	6
7.1	1.0	16	14
7.1	2.2	9	18
7.1	3.3	6	20

Fronds were cultured on media containing arginine and lysine of different concentrations. The contents of arginine and lysine in the tissues were determined after 3 long-days and the numbers of frond and flower were counted after 8 long-days.

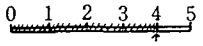
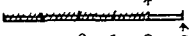
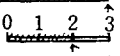
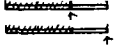
ratio above 20 may not cause any further drop in flower production, since at concentrations higher than 7.1×10^{-4} M exogenous arginine has been shown not to decrease FL % any more (cf. 5). Accordingly the progress

of the flower formation likely needs the ratio to be kept under 10. It is noted that this ratio remains apparently between 13 and 17 under short-day condition (Table V).

The next experiment gives a further support to the idea that when the ratio of arginine to lysine is under 10 flowering progresses normally. The fronds were cultured on arginine-containing medium for 2 or 4 long-days and were transferred to arginine-free medium for one more long-day. As reported previously, the former culture condition does not permit the duckweed to be induced, whereas the latter does. The in vivo ratio of arginine to lysine was determined before and after the arginine-free culture (Table VII). Only in the induced fronds the ratio was found to be equal to 10. Moreover, in the same table, the floral induction or the drop of the ratio to 10 is seen to involve a remarkable arginine degradation; lysine content being maintained nearly unaltered. A question will naturally be

TABLE VII

Contents of arginine and lysine in fronds fed with arginine for 2 or 4 long-days and transferred to arginine-free medium for one more long-day

Culture schedule (day)	Amino acid content (μmole leucine equiv./g F.W.)		Arginine/Lysine
	Arginine	Lysine	
	6.2	0.23	27
	2.3	0.23	10
	6.8	0.20	34
	4.4	0.29	15

See the explanation of Table III for symbols used. The arrow shows the time of analysis.

raised: if the activity of the duckweed to decompose certain crucial amino acids such as arginine may change periodically under long-day condition?

On the other hand, in Table V, exogenous arginine increased the in vivo arginine content three times that of control, leaving lysine content

TABLE VIII

Contents of arginine and lysine in fronds fed with L-lysine under long-day conditions

Lysine application period (hr)	Amino acid content (μmole leucine equiv./g F.W.)		Arginine/Lysine
	Arginine	Lysine	
Expt. 1 Control	2.4	0.21	11
11	2.6	2.1	1.2
Expt. 2 Control	2.1	0.24	9
24	2.2	2.4	0.9

Fronds were cultured on basal medium for 2 long-days before L-lysine (2.2×10^{-4} M) was added for designated hours under light.

unchanged. And, in Table VIII, exogenous lysine increased the in vivo lysine content by 10 times leaving the arginine content unmodified at all. Hence, the ratio of arginine to lysine will presumably not be determined by any direct interaction between the two amino acids in the tissues, but will be determined by some reaction(s) occurring probably under light condition.

DISCUSSION

It is widely known that a single amino acid added often affects considerably the growth and differentiation in higher plants, although amino acid mixtures may fail to do so (*13*, *14*). "Neomorph" in *Oenanthe aquatica* (*4*) and "frenching" in tobacco plants (*15*) are striking examples of malformation induced by amino acid. This kind of amino acid action can be reversed by simultaneous addition of other amino acid. Thus HARRIS (*1*) demonstrated with oat embryo that in certain pairs of amino acid, e.g., phenylalanine-tyrosine, lysine-arginine and valine-isoleucine, the inhibitory action of the former amino acid on growth could be reversed by the latter. It was reported also by SANSTED and SKOOG (*16*) that the inhibited growth of tobacco tissue by valine was alleviated by isoleucine. There are not only specific antagonistic pairs of amino acids, but also non-specific reversal by certain amino acids. HARRIS (*1*) found that amino acids producing marked inhibition were often antagonized by less inhibitory amino acids.

What is the mechanism of floral inhibition and its reversal in our duckweed by amino acids? The acids are likely unable to work as either metabolic intermediates or metabolic antagonists as suggested by the following findings:

1. The times of effective application coincided well for all of the inhibitory amino acids. It is therefore probable that the inhibition is related with some character(s) common to these amino acids.

2. Not the absolute but the relative amounts of amino acids, or, more exactly, the ratios in content of pair of antagonistic amino acids in the tissues, obviously played a decisive role in the inhibition.

3. The in vivo contents of arginine and lysine could be changed separately. That is, fronds apparently have no ability of regulating simultaneously the quantities of both of the antagonistic amino acids.

4. The inhibition of flowering by arginine could be reversed by lysine, and the inhibition by lysine could not be reversed by arginine (*5*).

In *Candida utilis*, a part of endogenous free amino acid pool has been suggested to be complexed with high molecular substances (perhaps protein) (*17*). ULRICH (*18*) has reported that in certain range of pH the activity of mitochondrial ATPase is inhibited by various amino acids.

Our temporal hypothesis will then be that the inhibition of flowering by amino acids may be caused by interaction(s) between free amino acids and component(s) (perhaps protein) of certain reaction system(s) involved

in flowering process, and the relative amounts of the amino acids associated with the component(s) may determine the activities of the latter. The component(s) in question need not be related directly to amino acid metabolism. It has relevantly been found that certain pairs of amino acids cannot affect the flowering. Further, the existence has been shown of such an amino acid as lysine which is capable of forming pairs non-specifically with a variety of amino acids. These may be useful clues to the clarification of the nature of presumed association between amino acids and the proteinous component(s). Whether, in general, the pools of low molecular substances can serve not only as metabolic intermediates but, as postulated above for amino acids, as controlling factors of the cellular activities should be investigated in future experiments.

As for the periodical change in sensitivity of duckweed to arginine, a possibility has been suggested that the cellular activity of decomposing arginine may change periodically. (See the explanation of Table VII in the text). This is also to be examined in detail.

Under short-day condition, the in vivo ratio of arginine to lysine is retained in the range which, under long-day condition, permits partially inhibited flowering. If a number of amino acids inhibit flowering process additively, the summation of their effects may evoke a perfect blockage of flowering as observed under short-day conditions.

At any rate, the present studies suggest strongly that the differentiation of granular body to flower which supposedly occurs after the lapse of the induction period may be unable to proceed before the in vivo states, such as the relative concentrations of free amino acids, are arranged under light condition to be favourable for the developmental process.

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