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Comparison of the Ability of Salicylic Acid and Ferricyanide to Induce Flowering in the Long-day Plant, *Lemna Gibba* G3

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ABSTRACT

Both salicylic acid and ferricyanide induce flowering in the long-day plant *Lemna gibba* L., strain G3 under 8- and 9-hour short days. In both cases the effect is daylength-dependent. Salicylic acid is ineffective on daylengths less than 8 hours and ferricyanide is ineffective on daylengths less than 5 hours. When both substances are given together a striking synergistic interaction is observed, and some flowering is obtained on daylengths as short as 3 hours. However, even with the optimal combinations the flower-inducing effect remains daylength-dependent.

Flowering of *L. gibba* G3 is inhibited under continuous light in both half-strength Hutner's medium (0.5 H), which contains 1.25 millimolar ammonium, and in ammonium-free half-strength Hutner's medium (NH_4^+-free 0.5 H). Salicylic acid is able to reverse the inhibition substantially in 0.5 H medium and to cause complete reversal in NH_4^+-free 0.5 H medium. By contrast, ferricyanide has no effect in 0.5 H medium and causes only a small reversal in NH_4^+-free 0.5 H medium. Flowering of *L. gibba* G3 is also inhibited under continuous light by copper. This inhibition is largely reversed by salicylic acid but ferricyanide has no effect.

Lemna gibba G3 is a qualitative long-day plant with a critical daylength of just under 10 h in E medium (1, 3, 4). Treatment with 3.2 μM salicylic acid leads to flower induction both on 10- and 11-h daylengths, where the controls show a low flowering response, and on 8- and 9-h daylengths, where the controls are completely vegetative (2). However, salicylic acid does not cause flowering on daylengths less than 8 h, so this effect is strongly daylength-dependent (4).

In 0.5 H[2] medium *L. gibba* G3 does not flower even under continuous light (8, 12). This inhibition was attributed to the ammonium present in Hutner's medium (8). While ammonium definitely does inhibit flowering, recent work has shown that in NH_4^+-free 0.5 H medium, flowering is still strongly inhibited (12). In both media the addition of salicylic acid results in profuse flowering (12).

Ferricyanide has been shown to cause substantial flowering of the short-day plant, *Lemna paucicostata* 6746, under continuous light (10, 14). It has also been reported that ferricyanide could stimulate flowering to a small extent in *L. gibba* G3 under 12-h daylengths (15).

In the present paper the influence of ferricyanide on flowering in *L. gibba* G3 has been investigated on short days with daylengths as short as 3 h, both alone and in combination with salicylic acid. In addition, ferricyanide and salicylic acid have been compared for their ability to stimulate flowering on 0.5 H and NH_4^+-free 0.5 H media and also for their ability to overcome the inhibition of flowering caused by copper (6).

MATERIALS AND METHODS

Plant Material. All work was done with the long-day plant *Lemna gibba* L., strain G3. It has been maintained in aseptic culture in this laboratory for over 10 years.

Culture Conditions. The media used in this study consisted of: (*a*) Hoagland-type media designated as M (5, 9) and E (1, 3) (differs from M medium only in the presence of 30 μM EDTA), both of which were prepared without the addition of $CuSO_4$ to minimize any inhibitory effect of copper on flowering (6); (*b*) 0.5 H medium which contains 1.25 mM NH_4NO_3 (7, 9); and (*c*) NH_4^+-free 0.5 H medium where 2.5 mM KNO_3 is substituted for the NH_4NO_3 (12). All media contained 1% sucrose and were prepared using deionized H_2O with a minimum resistance of 18 megohms that is produced by a Continental deionized water system. Salicylic acid and ferricyanide were added to the media prior to autoclaving. M and E media were adjusted to pH 4.6 and 0.5 H and NH_4^+-free 0.5 H media were adjusted to pH 6.2 prior to autoclaving. All media were sterilized by autoclaving for 14 min at 15 p.s.i. The plants were grown in growth chambers at a temperature of 28 ± 1 C. The light was provided by a mixture of four cool-white fluorescent lights and four 25-w incandescent bulbs with a combined fluence rate from 400 to 800 nm at plant level of 2.2–2.7 mw cm^{-2} as measured by a Gamma Scientific C3 spectroradiometer (corresponds approximately to 600–700 ft-c).

Experimental Procedure. All cultures were grown in 125-ml Erlenmeyer flasks with 50 ml of medium. The experimental flasks were covered with 50-ml glass beakers rather than cotton stoppers. The use of beakers has no effect on the flowering induced by long days or salicylic acid, but ferricyanide is much more effective when beakers are used, apparently because ferricyanide breaks down to release cyanide, and cyanide, which appears to be the active agent, is more easily lost when cotton stoppers are used (Tanaka and Cleland, unpublished). Cotton stoppers were used for all other flasks. Stock cultures were grown on E+ medium on a 9L:15D short-day regime (3). The procedure for obtaining the four-frond colonies used to start experiments was the same as previously described (1). A single four-frond colony was inoculated into each flask and, except where noted otherwise, all experiments lasted 11 days. By the end of the experiment the original four frond colony had grown into 40 to 200 or more fronds, except in the experiments shown in Table I where the combination of very short daylengths and growth inhibitory concentrations of salicylic acid and ferricyanide lead to final frond numbers as low as 15 to 20 in some cases. For those experimental treatments where daylengths other than continuous light were used, the plants received the experimental daylength for 8 days

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[2] Abbreviations: 0.5 H: half-strength Hutner's medium; NH_4^+-free 0.5 H: ammonium-free half-strength Hutner's medium; L: light; D: dark; FL%: flowering per cent; No. VF: absolute number of vegetative fronds.

Table I. *Influence of Salicylic Acid and Ferricyanide on the Flowering Response of L. gibba G3 when Cultured on E Medium on 3L:21D, 5L:19D, or 8L:16D Photoperiodic Regimes*

E medium is a Hoagland-type medium containing 30 μM EDTA and 1% sucrose. The 3L:21D treatment was given for 10 days followed by 3 days of continuous light. The 5L:19D and 8L:16D treatments were given for 8 days followed by 3 days of continuous light.

$K_3Fe(CN)_6$ Concentration	Photoperiodic Regime	Salicylic Acid Concentration (μM)							
		0		1		3.2		10	
		FL%	No. VF	FL%	No. VF	FL%	No. VF	FL%	No. VF
μM									
0		0	87.7 ± 1.9	0	58.0 ± 2.5	0	45.0 ± 3.6	0	50.3 ± 2.0
4	3L:21D	0	46.0 ± 2.0	4.8 ± 2.6	21.3 ± 2.4	3.7 ± 3.7	20.7 ± 2.4	0	23.3 ± 2.0
8		0	29.3 ± 2.0	8.1 ± 4.1	15.6 ± 1.5	4.1 ± 2.1	15.3 ± 0.9	0	17.7 ± 1.5
0		0	59.0 ± 1.2	0	37.0 ± 1.7	0	34.3 ± 0.9	0	36.7 ± 0.9
1	5L:19D	0	45.3 ± 1.3	0	32.7 ± 1.9	0	33.7 ± 2.3	0	31.3 ± 1.2
2		0	32.0 ± 2.1	7.0 ± 5.3	24.7 ± 2.8	15.1 ± 2.9	20.3 ± 4.4	0	23.7 ± 2.2
4		3.8 ± 1.9	20.3 ± 3.8	20.9 ± 4.7	13.7 ± 1.2	13.4 ± 6.7	12.3 ± 0.7	3.5 ± 3.5	15.3 ± 2.2
0		0	74.3 ± 1.2	0.5 ± 0.5	59.0 ± 1.5	2.1 ± 0.0	46.5 ± 0.5	2.1 ± 1.2	44.7 ± 2.6
1	8L:16D	0	63.3 ± 1.5	9.5 ± 4.8	36.0 ± 0.6	16.3 ± 3.8	35.3 ± 3.5	14.0 ± 2.3	33.3 ± 2.7
2		2.3 ± 1.2	47.3 ± 7.8	32.3 ± 2.0	22.3 ± 1.9	32.8 ± 1.7	17.0 ± 1.0	12.6 ± 2.6	28.7 ± 2.9
4		13.0 ± 1.5	21.7 ± 3.7	32.2 ± 2.8	11.3 ± 0.9	22.0 ± 7.4	15.0 ± 2.1	11.2 ± 1.9	18.7 ± 0.9

(in one case for 10 days) followed by 3 days of continuous light. Controls receiving 8 short days followed by 3 days of continuous light were always completely vegetative.

Flowering was evaluated both by the FL% and the No. VF in a culture (3). The No. VF is a measure of both growth and flowering, but the No. VF will almost always decrease when there is a significant increase in the level of flower induction. Three cultures were used for each treatment. Each experiment was repeated at least twice, but only the results of a single, representative experiment are presented. SE's were calculated for each treatment, and the results are presented as the mean plus or minus the SE of the mean.

RESULTS

Short-day flowering Induced by Ferricyanide and Salicylic Acid. Preliminary work showed that ferricyanide was capable of causing some flower induction on a 9L:15D short-day regime. Previous work had shown that when *L. gibba* G3 was grown in E medium the ability of salicylic acid to induce flowering was day-length-dependent, and no flowering was obtained on daylengths less than 8 h (4). To see if the influence of ferricyanide was also daylength-dependent, *L. gibba* G3 was grown in E medium on 8-, 5-, and 3-h daylengths with various concentrations of salicylic acid, ferricyanide or both substances together (Table I). In agreement with earlier work salicylic acid alone gave no flowering on daylengths less than 8 h. Ferricyanide alone proved to be somewhat more effective than salicylic acid but its effect was also daylength-dependent, since the flowering response decreased with decreasing daylength, and there was no flowering on a 3-h daylength. When salicylic acid and ferricyanide were given together, a striking synergistic interaction was observed, and some flowering was even obtained on a 3-h daylength. However, for any given combination the response was also clearly daylength-dependent.

Effect of Ferricyanide and Salicylic Acid on Flowering in 0.5 H and NH_4^+-free 0.5 H Media. Since ferricyanide proved even more effective than salicylic acid for inducing flowering when *L. gibba* G3 was grown on E medium under short days, ferricyanide and salicylic acid were also compared in their ability to reverse the inhibition of flowering under continuous light on 0.5 H and NH_4^+-free 0.5 H media (Table II). In agreement with earlier work (12) flowering in the controls was zero or virtually zero with the No. VF considerably higher in NH_4^+-free 0.5 H medium than in 0.5 H medium, and salicylic acid treatment resulted in a large flowering response on both media. Long-day controls grown on E medium for 11 days typically give a FL% of about 80 (1, 3). On NH_4^+-free 0.5 H medium salicylic acid is able to reverse completely the inhibition of flowering, although on 0.5 H medium salicylic acid is not quite as effective in reversing the inhibition, apparently due to the ammonium in the medium.

The effect of ferricyanide proved to be quite different from that of salicylic acid. On NH_4^+-free 0.5 H medium the flowering response at the optimum concentration was much less than that obtained with salicylic acid, as indicated by the smaller FL% and the higher No. VF. At still higher concentrations ferricyanide proved inhibitory to both flowering and growth (with 8 μM ferricyanide, total frond number was only 38 as compared to 119 with 10 μM salicylic acid).

In agreement with earlier results obtained with the short-day plant, *L. paucicostata* 6746 (13), addition of ammonium (0.5 H medium) completely inhibited the ability of ferricyanide to induce flowering and greatly reduced the growth inhibition due to ferricyanide.

Effect of Ferricyanide and Salicylic Acid on Reversing the Inhibition of Flowering by Copper. It is well established that low concentrations of copper will inhibit flowering of *L. gibba* G3 on continuous light (6). To test the ability of ferricyanide and salicylic acid to reverse this inhibition, plants were grown in M medium instead of E medium to avoid any possible complication due to the EDTA present in E medium (Table III). In the absence of added Cu, salicylic acid had essentially no effect except to reduce the growth rate slightly. When 4 μM Cu was added, flowering was completely inhibited and salicylic acid was able to reverse this inhibition to a substantial extent.

By contrast, ferricyanide was completely unable to reverse the inhibition of flowering caused by Cu. In the presence of Cu, ferricyanide caused only a slight reduction in the frond number, but in the absence of Cu growth was strongly inhibited with the total frond number being reduced from 165 for the minus Cu control to 46 for 4 μM ferricyanide. There was also a substantial decrease in the FL%. However, in *Lemna* there is always an initial number of fronds too large to be induced to flower at the start of an experiment (1, 3), and consequently there will always be some vegetative fronds in a culture at the end of an experiment. Thus, if the growth rate decreases substantially, the FL% will also decrease even if the level of flower induction remains constant. Since with 4 μM ferricyanide the No. VF is decreased, it would

Table II. *Influence of Salicylic Acid or Ferricyanide on the Flowering Response of L. gibba G3*

Lemna was cultured on 0.5 H or on NH_4^+-free 0.5 H under continuous light; 0.5 H contains 1.25 mM NH_4NO_3.

Treatment	0.5 H Medium		NH_4^+-free 0.5 H Medium	
	FL%	No. VF	FL%	No. VF
None	0	251.0 ± 8.9	1.1 ± 0.2	364.7 ± 6.8
1 μM $K_3Fe(CN)_6$	0	235.3 ± 1.9	15.8 ± 0.6	245.3 ± 2.9
2 μM $K_3Fe(CN)_6$	0	237.0 ± 7.0	23.1 ± 2.9	128.3 ± 7.4
4 μM $K_3Fe(CN)_6$	0	215.0 ± 7.6	10.0 ± 0.9	63.0 ± 2.1
8 μM $K_3Fe(CN)_6$	0	176.0 ± 4.0	0	38.0 ± 0.6
3.2 μM Salicylic acid	45.3 ± 6.0	65.0 ± 7.5	79.4 ± 1.3	38.7 ± 2.7
10 μM Salicylic acid	73.9 ± 0.4	23.0 ± 0.6	85.7 ± 0.7	17.0 ± 0.6

Table III. *Influence of Salicylic Acid or Ferricyanide on the Flowering Response of L. gibba G3*

Lemna was cultured on M medium with or without 4 μM copper under continuous light. M medium is a Hoagland-type medium with 1% sucrose.

Treatment	− Copper		+ 4 μM Copper	
	FL%	No. VF	FL%	No. VF
None	73.6 ± 2.2	43.3 ± 2.7	0	279.3 ± 8.4
1 μM $K_3Fe(CN)_6$	67.2 ± 4.5	46.0 ± 8.0	0	273.7 ± 7.2
2 μM $K_3Fe(CN)_6$	51.5 ± 1.0	43.0 ± 3.1	0	254.3 ± 3.0
4 μM $K_3Fe(CN)_6$	36.5 ± 4.1	29.0 ± 4.0	0	237.7 ± 4.2
8 μM $K_3Fe(CN)_6$	29.6 ± 4.5	20.3 ± 1.2	0	132.3 ± 5.2
3.2 μM Salicylic acid	77.0 ± 2.5	27.0 ± 2.9	63.7 ± 1.5	27.7 ± 1.8
10 μM Salicylic acid	75.7 ± 1.7	31.3 ± 2.2	55.7 ± 5.9	51.6 ± 5.8

appear that the level of flower induction is actually somewhat higher, despite the drop in the FL%.

DISCUSSION

This paper extends earlier work (4, 15) and demonstrates conclusively that ferricyanide is able to induce flowering in the long-day plant, *L. gibba* G3, on short days and that it is somewhat more effective than salicylic acid in this regard. In addition, we have shown that there is a substantial synergistic interaction for the induction of flowering when salicylic acid and ferricyanide are given together.

Earlier work has shown that the ability of salicylic acid to induce flowering in *L. gibba* G3 is strongly daylength-dependent and that the effect of salicylic acid treatment is to shift the critical daylength curve by about 2 h (4). The present work confirms those findings and indicates that the ability of ferricyanide to induce flowering in *L. gibba* G3 is also daylength-dependent, but in this case the critical daylength is shifted by about 5 h. Even when salicylic acid and ferricyanide are given together, the much larger flowering response that is obtained is also daylength-dependent since for any given combination of salicylic acid and ferricyanide the FL% decreases with decreasing daylength.

Both salicylic acid and ferricyanide cause a decrease in the growth rate of *L. gibba* G3 at concentrations effective in inducing flowering. However, with salicylic acid the fronds remain dark green and become swollen or gibbous (2), while with ferricyanide, the fronds remain relatively thin and at the higher concentrations show a tendency toward becoming light green or even yellow-green. The fact that the influence of salicylic acid and ferricyanide on flowering is not simply additive, but rather is synergistic, argues that the two substances are probably acting via different mechanisms in the stimulation of flowering. This conclusion is further supported by the striking differences observed in the ability of salicylic acid and ferricyanide to overcome the inhibition of flowering due to 0.5 H and NH_4^+-free 0.5 H media and also the inhibition caused by Cu. In each case salicylic acid is quite effective but ferricyanide has little or no effect.

Ammonium is known to inhibit flowering in *L. gibba* G3 (8, 12), and the fact that salicylic acid is more effective in NH_4^+-free 0.5 H medium than 0.5 H medium and ferricyanide has a small effect in NH_4^+-free 0.5 H medium but no effect in 0.5 H medium is probably attributable to the ammonium present in the 0.5 H medium. The inhibition observed in 0.5 H medium is not due just to ammonium since flowering is also strongly inhibited in NH_4^+-free 0.5 H medium that contains no ammonium. Work currently in progress suggests that plants growing under continuous light on NH_4^+-free 0.5 H medium are partially induced, but there is some block which prevents the expression of flowering. Ferricyanide is not very effective in removing this block. By contrast, salicylic acid is quite effective and at a concentration of 10 μM, flowering was as good or better than that normally obtained with long-day controls grown on E medium (12).

Ferricyanide has been shown to cause substantial flowering in the short-day plant *L. paucicostata* 6746 under continuous light (10, 14). Ferrocyanide and KCN are also quite effective (11), and available evidence strongly supports the conclusion that in each case the active agent is cyanide (11; Tanaka and Cleland, unpublished). Both ferrocyanide and KCN have been tested on *L. gibba* G3, and they proved to be as active as ferricyanide for the induction of flowering under short-day conditions. Thus by analogy it seems likely that cyanide is also the active agent for induction of flowering in *L. gibba* G3.

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