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Studies on the Growth and Flowering of a Short-Day Plant, *Wolffia microscopica*

II. Role of Metal Ions and Chelates*

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Summary. As found earlier, supply of EDTA was obligatory for both flowering and satisfactory vegetative growth in *Wolffia microscopica*. It is now shown that the metal affecting growth and flowering is most probably iron. Omission of Fe but not of Cu, Zn, Mn and B from the medium markedly affects vegetative growth. There exists also a strong interaction between EDTA and Fe, one being largely inactive in the absence of the other. When Fe-EDDHA is substituted for Fe-citrate and EDTA in the medium, no great effect is seen in vegetative growth, but flowering takes place even under continuous light. Studies with ^{59}Fe show that, in the medium containing Fe-EDDHA, Fe uptake is stimulated several-fold; this is apparently associated with the flowering condition.

Introduction

Earlier investigations on *Wolffia microscopica*, a duckweed, have shown that 1. by transferring plants from Bonner-Devirian to Hoagland's medium, the flowering response of this plant could be changed from quantitative to an obligate short-day type (Venkataraman et al., in press); 2. that whereas limited growth may continue even without EDTA, flowering of this plant occurs only in the presence of a chelate in the medium (Maheshwari and Chauhan, 1963); and 3. when EDTA + Fe-citrate are replaced with Fe-EDDHA flowering can be induced even under long days (Maheshwari and Seth, 1966). Since these observations indicated an important role of metal ions and of chelates in growth and flowering, it was considered desirable to probe further their mechanism of action.

Material and Methods

Material. The plant material was the same strain of *Wolffia microscopica* Griff. that was used in our previous experiments (Ventakaraman *et al.*, in press, and earlier).

* Abbreviations: EDTA = ethylenediaminetetraacetic acid; EDDHA = ethylenediamine-di-*o*-hydroxyphenylacetic acid.

The methods for raising aseptic cultures, media preparation, light and temperature conditions, determination of the multiplication rate (MR), and evaluation of flowering were also essentially the same as described in our earlier papers (Venkataraman *et al.*, in press). In the experiments reported in this paper only Hoagland's medium was employed.

Purification of Salts. For the studies on micronutrient deficiency and interaction of metal ions with chelating agents, the salts were further purified by recrystallization following the method of Munns and Johnson (1960).

To minimise contamination, the washed glassware was immersed in distilled water, autoclaved for 15 min at 10 lb/in^2, and rinsed with double-distilled water. For experiments concerning trace elements and estimations of Fe and Cu, the glassware was further rinsed with 0.5 N acetic acid and 1 N EDTA to ensure removal of heavy metals.

Estimation of Iron and Copper. Fe was estimated according to the method of Smith *et al.* (1952) which is based on the formation of a complex with *o*-phenanthroline, and Cu according to the method of Holmes (1945) in which a reaction occurs with dithizone. The assay in each case was carried out with 50-mg samples of dried fronds.

Uptake of Radioactive Iron. In experiments undertaken to compare the uptake of Fe in plants grown in Hoagland's medium containing EDDHA with those grown in EDTA, $^{59}FeCl_3$ (in dilute HCl) was used (3 μc/ml of medium), in addition to non-radioactive Fe added as ferric citrate. The total Fe concentration of the solution was 1 μg/ml. After adding radioactive Fe, the pH of the medium was lowered to 2 in order to dissociate the existing non-radioactive iron-chelate complex, and readjusted to 5.5. After 6 or 7 days of culture in the radioactive media, the plants were harvested and washed thoroughly with distilled water. 200 plants were taken out randomly from 8 replicate cultures, dried on aluminium planchets, and their radioactivity determined in a Geiger-Müller counter.

Results

Early during our investigations we observed that flowering in *W. microscopica*, despite short-day treatment, occurred only when EDTA was present in the medium. Some growth took place in the absence of EDTA but not too satisfactorily. Thus, it seemed to us that some common factor was affecting both vegetative growth and flowering, although more specifically the latter process. With this idea, a detailed study was made of the effects of chelates and metal ions on both of these processes, in an attempt to identify the metal ion responsible for these effect.

1. Growth

Effect of Concentration and Deficiency of Various Trace Elements. The results with EDTA suggested two possibilities: 1. In the unchelated medium some metal ion may be toxic to the growth of plants, or 2. in such a medium some essential metal ion may not be sufficiently available to the plant. To test for possible toxicity of the medium, experiments were carried out on the effect of dilute Hoagland's medium on the

multiplication rate of the plants. Half-diluted medium was just as good for growth as the normal medium, at least over 8 days. However, medium diluted one-fourth and one-eighth was decidedly inferior. It was clear that dilution of the medium did not improve growth in any way; in fact, higher dilutions caused an appreciable reduction in multiplication rate, rendering the idea of a metal toxicity improbable.

To test the alternative idea, i.e. that EDTA prevented precipitation or facilitated the entry into the plant of some ion essential for growth, six sets of media were prepared and one trace element at a time was omitted from the medium. Before inoculation, the plants were washed thoroughly for 4—5 hr. with the medium in which they were to be grown, and only a single colony was inoculated in each culture tube to reduce any carry-over effect. Deficiency symptoms appeared in two treatments: omission of Mn or of Fe resulted in chlorosis and decline of the growth rate within a few days. In contrast, omission of Cu^{++}, Mo, Zn^{++} or B resulted neither in any visual deficiency symptom nor in any significant change in the multiplication rate during the experimental period (ca. 12 days). These results led to the idea that either Fe^{+++} or Mn^{++}, or possibly both, were playing a critical role in the growth of *W. microscopica* and that EDTA might act by chelating one or both of these ions.

Effect of Other Chelating Agents. Apart from EDTA, a variety of other chelating agents are available; they are known to differ in their affinity for different ions. We chose citric acid, tartaric acid, EDDHA, Fe-DTPA (ferric diethylenetriaminepentaacetate) and Fe-EDDHA (iron salt of EDDHA) for further tests. The chelating agents showed differences amongst themselves both in regard to the optimal concentration required for growth, and the maximal growth achieved (Fig. 1). Citric and tartaric acid supported growth very little if at all.

Since ferric citrate itself is a metal-chelate complex, experiments were conducted employing high levels of this compound. Fe-citrate at 10^{-4} M did indeed elicit better growth than at 1.1×10^{-5} M (the normal level in the medium), although it did not match the effect of supplying Fe-citrate and EDTA together. The metal chelate, Fe-DTPA, also stimulated growth. But the most pronounced response was obtained with EDDHA in combination with the standard amount of Fe-citrate in the medium. The multiplication rate was highest at 10^{-7} M, a concentration about 500-fold less than that required in the case of EDTA. The metal-chelate complex, Fe-EDDHA, was also found to be satisfactory for growth. Interestingly, unlike EDTA which had a comparatively well-defined optimum at 5×10^{-5} M EDTA, there was no sharp optimum for Fe-EDDHA; the plants showed excellent growth between the concentrations of 10^{-5} and 10^{-3} M.

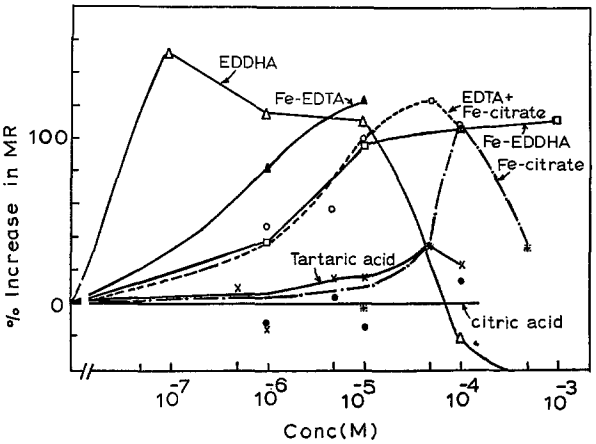

Fig. 1. Comparison of the effects of different chelating agents (relative to citric acid) on multiplication rate (*MR*)

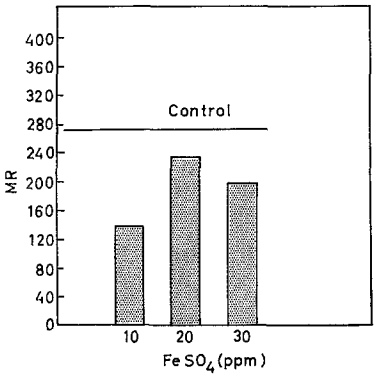

Fig. 2. Effect of varying concentrations of $FeSO_4$ on multiplication rate (*MR*) of plants grown in Hoagland's medium without EDTA. For comparison, the MR obtained in the normal medium containing EDTA and ferric citrate is also shown

Effect of $FeSO_4$. Since the results with the chelates — especially the iron chelates — implicated a role of Fe in growth, an experiment was set up in Hoagland's medium containing between 0 and 30 mg/l $FeSO_4$ without any chelating agent (Fig. 2). $FeSO_4$, which had its optimal effect at 20 mg/l, supported growth of *W. microscopica* for at least 15 days. However, the multiplication rate was lower than that in the medium containing EDTA and ferric citrate.

Interaction of Metal Ions with EDTA. Yet another approach towards determining the role of EDTA was to observe the effect of interaction of

different metal ions with EDTA on growth. The experiments were done employing a modified Hoagland's medium containing various concentrations of EDTA, and of Fe^{+++}, Mn^{++}, Zn^{++} and Cu^{++} (these metals were selected as EDTA is known to have a strong affinity for all of them). Besides the standard concentration (see Venkataraman *et al.*, in press) of each metal ion, two other concentrations — one higher than the standard and another lower — were also included, as well as a treatment where the metal ion in question was omitted altogether.

Fe^{+++} and EDTA as well as Mn^{++} and EDTA showed a positive interaction; vegetative growth and vigour improved when both EDTA and the metal ion were present. However, plants showed chlorosis and later turned brown in all cultures from which either EDTA or the metal ion was absent. On the other hand, no significant interaction was observed between EDTA and Zn^{++} or between EDTA and Cu^{++}, except that the toxicity caused by excess of either Zn^{++} or Cu^{++} could be overcome by high concentrations of EDTA. The plants remained green and healthy in all concentrations of EDTA employed with Zn^{++} and Cu^{++}.

These results again narrowed the choice to either Fe or Mn, or both, as limiting factor(s) in growth and flowering of *W. microscopica*.

2. Flowering

In parallel with the experiments on vegetative growth, the effect of different chelates was examined on flowering.

Effect of Fe-EDDHA and EDDHA. Most of the chelates tested behaved in the same way as EDTA, i.e. the presence of a chelate permitted flowering, but only when short days were also provided. However, we observed an unexpected response to Fe-EDDHA and EDDHA.

In media containing 10^{-5} to 10^{-3} M Fe-EDDHA, *W. microscopica* flowered under long days, the maximal flowering response being obtained at 10^{-4} M (Fig. 3a). There was no flowering at 10^{-7} and 10^{-6} M Fe-EDDHA although the plants were healthy. When a narrower range of concentrations between 10^{-5} and 10^{-4} M was tested, profuse flowering occurred under long-day conditions in all concentrations (1.1—4.5×10^{-5} M) employed, but the maximum (60%) was attained at 2.3×10^{-5} M (10 mg/l). Control plants grown in the regular medium containing EDTA and Fe-citrate showed no flowering under long days even though they showed a high rate of multiplication.

When both short days and Fe-EDDHA were provided, 100% flowering was observed (as against 50% flowering in the standard medium containing EDTA + Fe-citrate) with all concentrations of Fe-EDDHA employed.

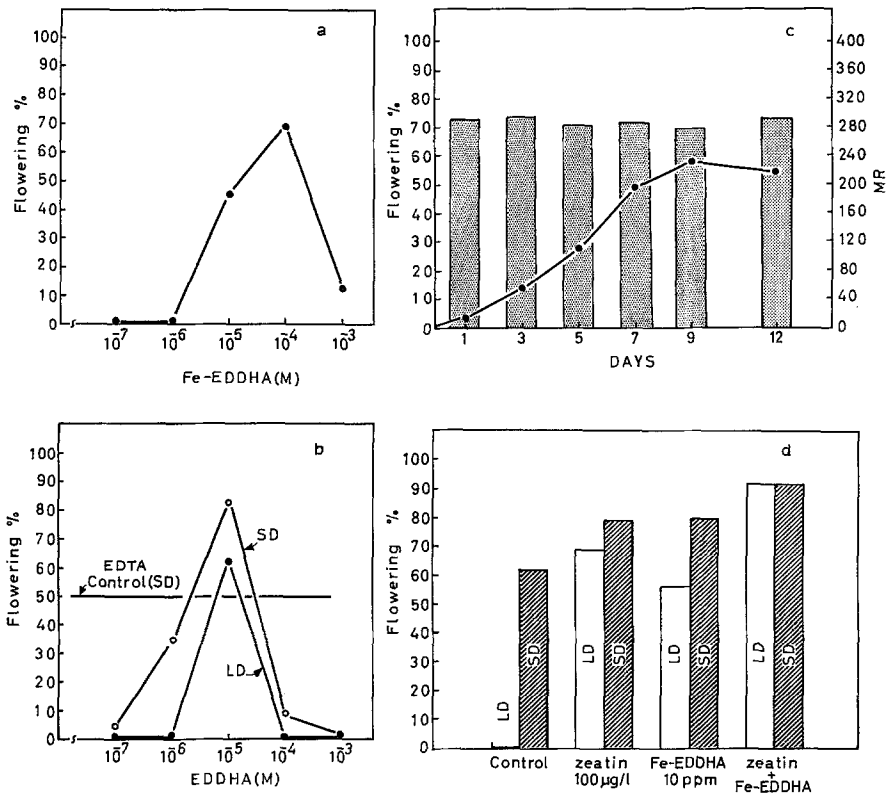

Fig. 3. a Effect of Fe-EDDHA on the flowering of plants grown under long days. b Effect of EDDHA on flowering under long (*LD*) as well as short (*SD*) days. For comparison, the level of flowering obtained after short-day treatment in the normal medium containing EDTA and ferric citrate is also shown. c Flowering of plants grown in medium containing 10 mg/l Fe-EDDHA under long days as a function of time. Vertical bars represent the multiplication rate (*MR*). d Effect of zeatin on flowering in media containing 5×10^{-5} M EDTA or 10 mg/l Fe-EDDHA. Fe-EDDHA, when present, substituted for both EDTA and Fe-citrate

The free acid EDDHA, too, induced flowering under long days, but in contrast to Fe-EDDHA it did so only at 10^{-5} M. Under short days, however, EDDHA caused flowering over a wider range of concentrations. Even at 10^{-6} M 34% flowering was observed, and at 10^{-5} M, where 62% plants flowered under long days, short-day treatment raised the percentage to 82 (Fig. 3b). Higher concentrations of EDDHA inhibited flowering, but some flowering was observed even at 10^{-4} M.

Kinetics of Flower Induction by Fe-EDDHA. In the experiment illustrated in Fig. 3c, plants were transferred under long days from the

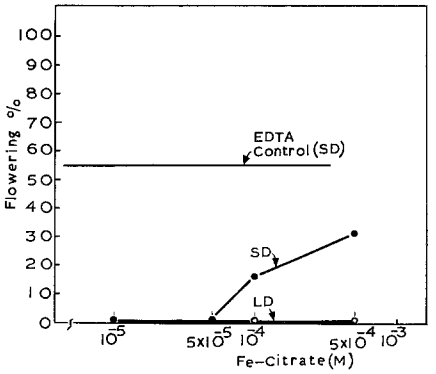

Fig. 4. Flowering of plants under long (*LD*) and short (*SD*) days in media containing varying levels of ferric citrate. The SD treatment consisted of two cycles of 6 hr. light + 18 hr. darkness each. For comparison, the level of flowering under short days in control medium containing EDTA and Fe-citrate is also shown

Fe-EDDHA medium to a medium containing EDTA and Fe-citrate; the transfer was done after 1, 3, 5, 7 or 9 days of growth. Flowering was initiated in plants grown in the Fe-EDDHA medium even for a single day (as expected, in the EDTA + Fe-citrate medium the plants remained vegetative).

Although some variation has been encountered among different experiments, the percentage of flowering continued to rise with the number of days on Fe-EDDHA, until it reached 100%.

Interaction between Fe-EDDHA and Zeatin. We have earlier reported induction of flowering in *W. microscopica* under long days by zeatin (Maheshwari and Venkataraman, 1966; Venkataraman *et al.*, in press) and it was of interest to know whether there was any interaction between zeatin and Fe-EDDHA. In the EDTA + Fe-citrate medium, as expected, there was no flowering under long days. In the media containing zeatin or Fe-EDDHA alone the percentage of flowering plants was 69 and 56, respectively (Fig. 3d). However, when Fe-EDDHA and zeatin were provided simultaneously, the percentage was 92. Clearly, the two compounds supplied together induce more flowering than when used singly. This holds also under short days although the differences here are less striking.

Effect of Ferric Citrate. Both EDTA and EDDHA are known to have a high affinity for Fe and if EDTA and EDDHA promote flowering by virtue of this property, one might expect to obtain the same effect by provision of higher levels of Fe-citrate alone (i.e. without another chelate). At high levels of Fe-citrate, plants do indeed flower without

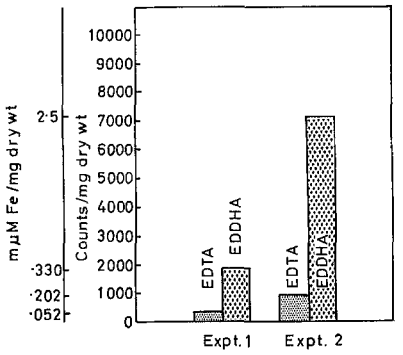

Fig. 5

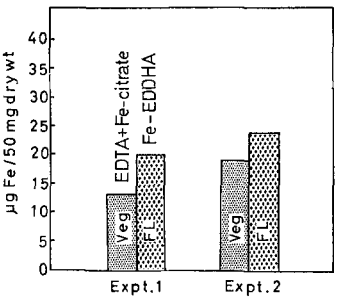

Fig. 6

Fig. 5. Iron content of plants grown in media containing EDTA + Fe-citrate, or Fe-EDDHA, under long days

Fig. 6. Comparison of uptake of radioactive iron (^{59}Fe) by plants grown in Hoagland's medium containing EDTA with that by plants grown in EDDHA under long days; counts are per minute

any additional chelate, flowering being linear between 5×10^{-5} M and 5×10^{-4} M (Fig. 4), although it was considerably less than that in the control medium containing EDTA + Fe-citrate. Higher concentrations of Fe-citrate were not tried on account of partial precipitation in the medium.

Effect of Other Metal Ions on Flowering. Since in *L. perpusilla* flowering can be induced under long days in a purified medium by providing excess copper (Hillman, 1962), an experiment was set up with *W. microscopica* on media containing 0, 10^{-6}, and 10^{-5} M $CuSO_4$ to examine any effects of Cu on flowering under long days. In the absence of EDTA, the high concentrations of Cu^{++} proved toxic for plants. In the presence of EDTA, the plants multiplied normally at all concentrations of Cu tested, but failed to flower. Thus, increase in the level of Cu^{++} does not induce flowering under long days.

Changes in Content and Uptake of Iron Following Transfer to Fe-EDDHA Medium. Since several observations recorded above — particularly those concerning flowering on long days by Fe-EDDHA — provide strong evidence for the chelation of Fe as a factor in flower induction, a study was made of the changes in endogenous levels of Fe following treatment with Fe-EDDHA. Total endogenous Fe was estimated after plants had grown for 10 long days in the two media. Plants provided with Fe-EDDHA had 25—50% more Fe than those grown in medium containing EDTA and Fe-citrate (Fig. 5).

Experiments with radioactive iron (^{59}Fe) confirm the observation that the level of Fe increases during flower induction. Fig. 6 shows that plants grown in the EDDHA medium take up about 500—700% more iron than those grown in EDTA medium.

Changes in the Endogenous Copper Levels. A study was also made of the changes in the endogenous levels of Cu. In all experiments the levels of endogenous Cu in vegetative and flowering plants showed a difference. However, contrary to the results observed with Fe, the level of Cu decreased by 15—30% in flowering plants.

Discussion

That flowering is sometimes profoundly influenced by chelates has been known for sometime through the work of Hillman (1961a, b, c) on other duckweeds, namely, *Lemna perpusilla* and *L. gibba*. In the normal medium, *L. perpusilla* flowers both under short and long days, but in the presence of EDTA flowering occurs only under short days. According to Hillman (1962) EDTA brings about its influence by chelation of excess Cu. This conclusions has been arrived at from two basic experiments: 1. After recrystallizing the salts and purifying the medium flowering occurred only under short-day conditions, an effect normally observed only on the addition of EDTA; and 2. by adding 0.05—10 μmoles/ltr Cu to such a medium, the response of the plant could again be made day-neutral.

Our work on *Wolffia microscopica* provides new evidence for control of flowering by chelates. However, our results are substantially different from those of Hillman. The most important point is that in *W. microscopica*, EDTA acts on flowering in a promotive rather than a restrictive manner. In fact, in the absence of EDTA flowering is totally suppressed. Furthermore, EDTA does not seem to bring about its effect by removing a toxic ion, but by facilitating the entry of iron — an element essential for plant growth. It appears to us that Fe is in some way specifically required for flower initiation and, under threshold conditions or when availability of Fe is limited, only vegetative growth may proceed. These conclusions are supported by several pieces of evidence.

Deficiency as well as interaction experiments showed that Fe and Mn are the two elements most critically required for the growth of *W. microscopica*. Experiments in which, instead of providing a chelating agent like EDTA or EDDHA, the levels of different micronutrients are increased, indicate that only when the level of iron in increased do the plants show normal growth and flowering, but not with Cu, Zn and Mn.

Perhaps the most interesting observation in the present investigations concerns the effect of Fe-EDDHA on flowering. Replacement of EDTA +

Fe-citrate by Fe-EDDHA results in flowering even under normally non-inductive conditions, i.e. under long days in Hoagland's medium. This change-over from a medium containing EDTA + Fe-citrate to another containing Fe-EDDHA has hardly any effect on growth. Analysis of the chlorophyll content also has failed to show any marked difference between plants grown in different media. If anything, there is less chlorophyll in plants grown in Fe-EDDHA-containing medium. Thus, the experiments with Fe-EDDHA help us to separate the growth and the flowering responses. Where EDTA and Fe-citrate were employed one could still argue that flowering is a consequence of improved growth of the plants; with Fe-EDDHA, however, it is evident that the effects are more direct on flowering.

Studies with Fe-EDDHA implicate Fe more closely in flowering. EDDHA has a higher stability constant (> 33) for Fe than has EDTA (25). The data presented in Fig. 3b show clearly that EDDHA may exercise its effect even at lower concentrations, and it seems that under these conditions EDDHA would be bound almost entirely to Fe. This can also be judged by the fact that media in which EDDHA is included are pink-red, having an absorption maximum at 490 mμ which is the same as that of pure Fe-EDDHA (see Kroll *et al.*, 1957; Frost *et al.*, 1958).

The estimates of Fe also point to the same conclusion. The Fe content of plants grown in Fe-EDDHA medium is about 25—50% higher than of those grown in the medium containing EDTA and Fe-citrate. The experiments with radioactive Fe show that when plants are transferred to a medium containing EDDHA, there is a dramatic increase in the uptake of Fe — to almost 700% of that in plants grown in the EDTA medium under comparable conditions.

A role of Fe in flowering has also been pointed out in some earlier work, namely on *Xanthium pennsylvanicum* by Smith *et al.* (1957); it was shown that among the various micronutrients, deficiency of Fe is the most deleterious for flowering. Thus, all evidence strongly indicates that the chelating agent brings about its effect primarily by binding of Fe. The biochemical role of Fe remains of course to be investigated; obvious lines of further enquires are the intracellular site of accumulation of Fe; the fate of the latter; the possibility of activation or synthesis of any Fe-containing enzyme; and the effect of Fe on phytochrome conversion. Lastly, one cannot ignore indirect effects of Fe accumulation on metabolism of other ions; thus, it is possible that the decrease in Cu content on Fe-EDDHA medium may be due to such an influence.

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