Plant Cell Physiol. 27(5): 919–924 (1986)

A Comparison of the Effects of Chelates, Salicylic Acid and Benzoic Acid on Growth and Flowering of *Spirodela polyrrhiza*

J. P. Khurana and S. C. Maheshwari

Unit for Plant Cell and Molecular Biology, Department of Botany, University of Delhi, Delhi-110007, India

Flowering in the genus *Spirodela*, whether in the laboratory or in nature, has been observed only rarely. In this communication, the growth and flowering behaviour of a local isolate of *S. polyrrhiza*, strain SP_{20}, is being reported. The presence of a chelate, such as EDTA, is obligatory for satisfactory vegetative growth of *S. polyrrhiza* SP_{20}. An optimal flowering response is obtained, however, only when compounds such as EDDHA, a phenolic analog of EDTA, or benzoic acid are supplied. Flowering, so induced, is not influenced by the length of the photoperiod. Flowering fronds become gibbous and both EDDHA and benzoic acid also enhanced anthocyanin content.

This investigation has also revealed that salicylic acid, which is known to have chelating properties itself, induces flowering in this duckweed only in the simultaneous presence of EDTA, in the nutrient solution.

Key words: Benzoic acid — EDDHA — Flowering — Salicylic acid — *Spirodela polyrrhiza*.

Spirodela polyrrhiza, also called 'greater duckweed', is the largest in size amongst the duckweeds and is world-wide in distribution. Nevertheless, like other duckweeds, *Spirodela* flowers infrequently in nature and there are only a few reports where flowering has been observed in the laboratory. In *S. polyrrhiza*, Lacor (1968) could obtain only 10% flowering in the presence of casein hydrolysate in the nutrient solution and Wolek (1974) reported flowering in strain 7401 without any quantitative data. The first report, where a substantial degree of flowering was observed in *Spirodela punctata* (formerly designated as *S. oligorrhiza*), came from Kandeler's laboratory—flowering could be induced in the presence of SA as well as EDDHA (Scharfetter et al. 1978). This was followed by a short communication from our laboratory, wherein induction of flowering in *S. polyrrhiza* SP_{20} by SA was demonstrated (Khurana and Maheshwari 1980).

We present here data to show control of flower initiation in this local duckweed strain by EDDHA and benzoic acid.

Materials and Methods

Strain SP_{20} of *Spirodela polyrrhiza* was collected from the lake at the Ghana Bird Sanctuary, Bharatpur, India, in December 1977. Aseptic cultures of *S. polyrrhiza* SP_{20} were maintained in modified Bonner-Devirian medium, i.e. containing the macro-nutrients of Bonner-Devirian

Abbreviations: BA, benzoic acid; EDTA, ethylenediaminetetraacetic acid; EDDHA, ethylenediamine-di-*o*-hydroxyphenylacetic acid; MR, multiplication rate; SA, salicylic acid.

(1939) medium and the micro-nutrients of Heller's (1953) medium. This nutrient medium was supplemented with 10^{-4} M EDTA and 1% sucrose. The pH of the medium was adjusted to 5.5 before autoclaving at 1.08 kg cm^{-2} for 15 min. All experimental cultures were started with a single 3-frond colony in 250 ml Erlenmeyer flasks (containing 100 ml nutrient solution), under photoperiodic schedules of 16 h light and 8 h darkness. The temperature was maintained at 26 ± 2°C during day time and at 22 ± 1°C during darkness. Light was provided from a mixed bank of cool daylight fluorescent tubes (Philips TL 65-80W/54 RS, 6,800°K) and tungsten bulbs (Philips Argenta, 100 W). The irradiance in the spectral range between 400 and 750 nm, measured with the help of a Model SR spectroradiometer (Instrumentation Specialties Co., Lincoln, Nebraska), varied from 10.0 to 10.5 W m^{-2}.

MR was calculated according to Clark (1925) as $MR=(\log_{10}Fd-\log_{10}Fo)\ 1{,}000/d$, where Fo is the original number of fronds and Fd is the frond number on day d. Flowering percentage was calculated by dividing the number of flowering fronds by total number of fronds and multiplying by 100. All flowering stages were taken into consideration. For each replicate culture flask, percentage flowering was calculated from about 60–100 plants. The percent values so derived from three culture flasks were averaged.

Anthocyanins were extracted in 1% acidic methanol and absorbance read at 529 nm. Total chlorophyll content was determined according to the procedure of Smith and Benitez (1955).

The experiments were repeated at least once, and usually several times, but data of only a representative experiment are presented here.

Results

Effect of EDTA—In the basal Bonner-Devirian medium, without any conventional chelator, it was noticed that plants are not too healthy; fronds were thick and yet chlorophyll content was rather low (visual observation). Supplementing the medium with EDTA resulted in healthy growth of the fronds—plants not only multiplied rapidly but were also greener. The optimal level of EDTA was 10^{-4} M, where ca. 50% increase in MR over the control plants was observed (Fig. 1).

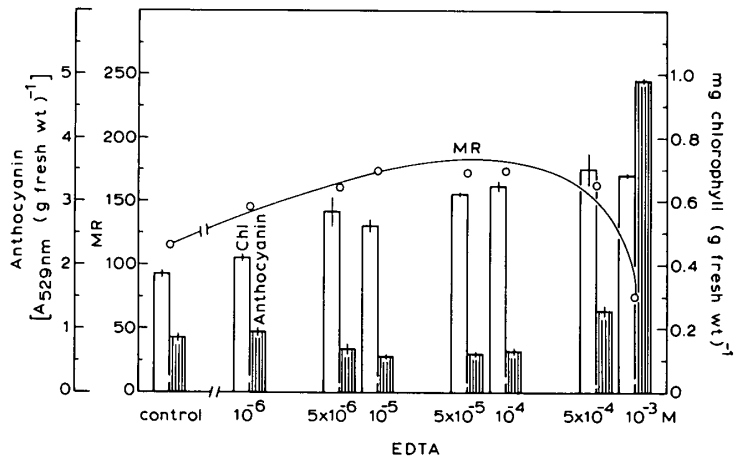

Fig. 1 Effect of EDTA on multiplication rate (MR), total chlorophyll (Chl) and anthocyanin contents of *Spirodela polyrrhiza* SP_{20}, under a photoperiodic schedule of 16 h light and 8 h darkness, in the modified Bonner-Devirian medium. A three-frond colony was inoculated in each flask from an exponentially growing 'stock' culture. MR was calculated and pigments estimated in ten-day-old cultures. Curve represents the MR (mean of three replicates). Vertical bars represent Chl and anthocyanin contents (mean of two replicates; separate extractions), and vertical lines the extent of absolute variation in the replicates.

An increase in total chlorophyll content was observed with increase in the level of EDTA and anthocyanin content decreased marginally at levels of EDTA greater than 5×10^{-6} M (Fig. 1). A rather marked increase in the level of anthocyanin was observed at 10^{-3} M, where MR declined considerably (Fig. 1). Regardless of the effects on growth, however, flowering could not be initiated at any concentration of EDTA between 10^{-6} and 10^{-3} M.

Effect of EDDHA—EDDHA was found to be quite satisfactory for growth at lower levels, i.e. 10^{-6}, 10^{-5} and 2×10^{-5} M, but higher concentrations were distinctly inhibitory (Fig. 2). EDDHA, at all levels tried, caused an increase in chlorophyll as well as anthocyanin content, except at 5×10^{-4} M, where growth was otherwise retarded (Fig. 2). Besides this, at 10^{-4} and 5×10^{-4} M levels of EDDHA, fronds became gibbous and their size was considerably reduced.

The effect of EDDHA on flowering is really noteworthy. More than 55 per cent fronds flowered, under a photoperiodic schedule of 16 h light and 8 h darkness, although at rather high molarity, i.e. 5×10^{-4} M (Fig. 2). In some experiments, however, as much as 75 per cent flowering could be observed, as would be obvious from the results described below. Experiments were also designed to determine whether EDDHA-induced flowering may be influenced by different photoperiods. However, the length of photoperiod did not have any significant effect and flowering was nearly uniform under all schedules, except in the plants kept in continuous light where the flowering percentage was somewhat lower (Fig. 3).

Effect of salicylic acid—Earlier, we had reported that SA induces profuse flowering in *S. polyrrhiza* SP_{20}, in the simultaneous presence of 10^{-4} M EDTA in the modified Bonner-Devirian

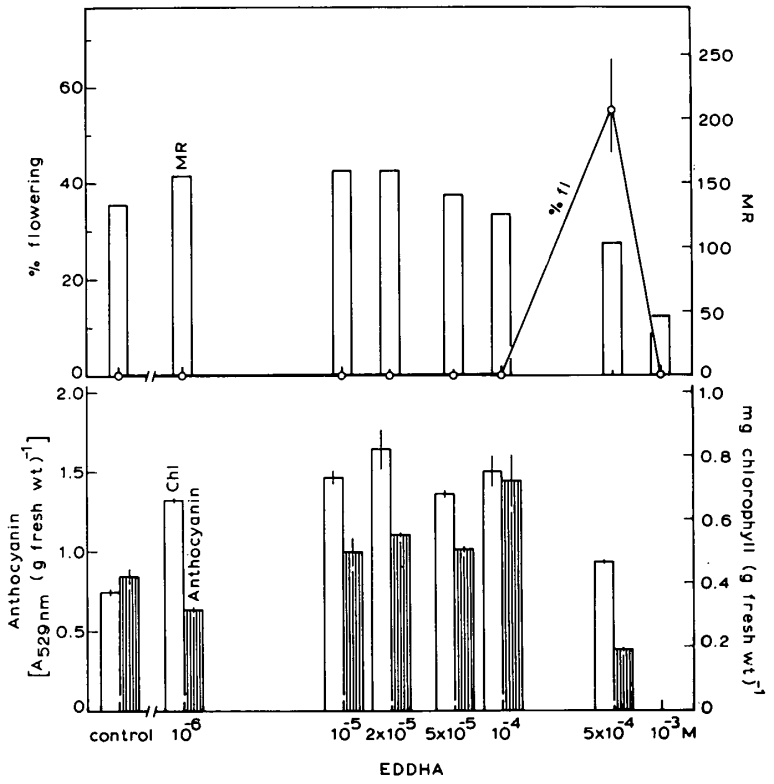

Fig. 2 Effect of EDDHA on multiplication rate (MR), total chlorophyll (Chl) and anthocyanin contents, and flowering of *Spirodela polyrrhiza* SP_{20}, under photoperiods of 16 h light and 8 h darkness. Experimental cultures were initiated in the presence of different levels of EDDHA in the modified Bonner-Devirian medium, without EDTA. MR was calculated and estimation of pigments was done in 11-day-old cultures. Fronds were analysed for flowering after 19 days. Curve represents the flowering percentage (each value plotted is the mean of three replicates). For more details see legend to Fig. 1.

medium (Khurana and Maheshwari 1980). SA-induced flowering, too, was not influenced by length of the photoperiod.

The potency of SA for floral induction in *Spirodela* in the presence of EDTA was easily re-established. But since SA is known to chelate metal ions by itself, it was of interest to determine whether SA alone—in the absence of the conventional chelating agent, EDTA—would be effective for growth and, also, induce flowering in *S. polyrrhiza* SP_{20}. Plants were raised in the medium supplemented with filter-sterilized SA (at levels varying from 10^{-7} to 10^{-4} M), in the absence of EDTA. But, unlike EDTA and EDDHA, SA could not support a healthy growth and plants remained pale green throughout, at all the levels tried, and no flowering could be observed; cultures were kept under photoperiodic cycles of 16 h light and 8 h darkness for 14 days. Higher concentrations (above 10^{-6} M) of SA markedly retarded growth—MR was ca. 120, 110 and 15, at 5×10^{-6}, 10^{-5} and 5×10^{-5} M levels, respectively, as compared to 150 in untreated controls, in the absence of EDTA. Growth was completely arrested at 10^{-4} M level of SA.

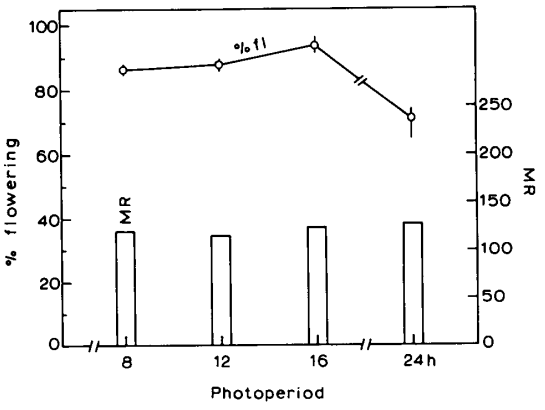

Fig. 3

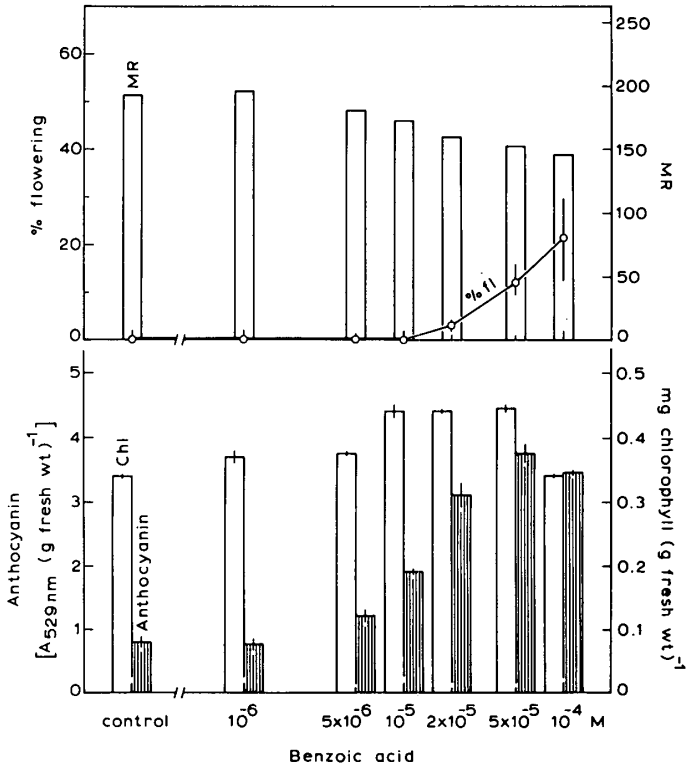

Fig. 4

Fig. 3 Influence of daylength on the ability of EDDHA to induce flowering in *Spirodela polyrrhiza* SP_{20}. Experimental cultures were raised in the presence of 5×10^{-4} M EDDHA in the modified Bonner-Devirian medium, devoid of EDTA. Four separate sets of cultures were prepared and subjected to different photoperiods. The duration of the experiment was 15 days, with the first two and the last three days of 16 h light and 8 h darkness, for all treatments. For more details see legend to Fig. 1 and 2.

Fig. 4 Effect of benzoic acid on multiplication rate (MR), total chlorophyll and anthocyanin contents, and flowering of *Spirodela polyrrhiza* SP_{20}, under photoperiodic schedules of 16 h light and 8 h darkness. Experimental cultures were initiated in the modified Bonner-Devirian medium, in the presence of 10^{-4} M EDTA. BA (filter-sterilized) was added to two-day-old cultures. The estimation of the pigments and the MR was done after seven days, and fronds analysed for flowering 15 days after BA treatment. The values for the MR and flowering represent the mean of three replicates and that of pigments, the mean of two replicates. For more details see legends to Fig. 1 and 2.

Effect of benzoic acid—For its effect on MR, total chlorophyll content and anthocyanins, BA proved to be almost equally potent as SA (the results with SA were reported earlier by us; Khurana and Maheshwari 1980). BA also induced flowering; however, the percentage of flowering did not exceed 20% (Fig. 4). At concentrations effective for flowering, plants also became gibbous. Increase in the levels of anthocyanins as well as total chlorophyll was also monitored. An increase of only 30% was recorded in chlorophyll content, whereas anthocyanins increased markedly—more than 4-fold increase occurred over controls, with 5×10^{-5} M BA (Fig. 4).

Discussion

It is evident from the results presented above that strain SP_{20} of *S. polyrrhiza* remains vegetative when grown in basal medium, with or without the presence of EDTA, and it does not flower under any of the photoperiodic schedules tried. For satisfactory vegetative growth, it has an obligatory requirement of a chelate, such as EDTA—in this respect, *S. polyrrhiza* SP_{20} resembles *Wolffia microscopica*, where EDTA also ensures a normal, healthy vegetative growth (Seth et al. 1970, Khurana 1982).

EDDHA had a marked stimulatory effect on flowering in *Spirodela*. Flowering was, however, not influenced by the photoperiod and it could be *induced* even under continuous light. This effect of EDDHA on flowering in *S. polyrrhiza* SP_{20} is in conformity with earlier reports where it has been shown to influence flowering (e.g. *W. microscopica*, Seth et al. 1970; a strain of *Lemna paucicostata*, Gupta and Maheshwari 1970; *L. gibba* G_3, Pieterse et al. 1970; *Spirodela punctata*, Scharfetter et al. 1978; *L. paucicostata* 151, Watanabe and Takimoto 1979 and *L. paucicostata* LP_6, Khurana 1982).

The response of *S. polyrrhiza* SP_{20} to EDDHA, with respect to various developmental processes (such as evocation of flowering, size of the fronds, gibbosity, multiplication rate, total chlorophyll content) is nearly identical to an earlier report on *S. punctata* (Scharfetter et al. 1978). However, the level of EDDHA required for evocation of flowering in strain SP_{20} (employed in the present investigation) was many-fold higher than required for *S. punctata* (reported earlier by Scharfetter et al. 1978). This may be due to the differential requirement for nutrients, as well as growth behaviour of these two strains of *Spirodela*.

Benzoic acid, though unlike SA, it has negligible chelating activity, nevertheless, not only did it influence flowering but also enhanced chlorophyll and anthocyanin contents (see Fig. 4) and induced gibbosity in *S. polyrrhiza* SP_{20}. The results obtained with BA are fairly comparable with SA (reported earlier; see Khurana and Maheshwari 1980) but, BA is less effective than SA, so far as intensity of flowering response of strain SP_{20} is concerned—only 20% flowering could be achieved with 10^{-4} M BA, whereas 70% flowering was obtained with equimolar level of SA.

The results presented in this communication show that SA (a known chelator), unlike EDTA and EDDHA, does not promote vegetative growth of *S. polyrrhiza* SP_{20}. But for flowering (only in the simultaneous presence of 10^{-4} M EDTA), it is many times more effective than these conventional chelates. This is a situation similar to the one exhibited by *W. microscopica* where the presence of EDTA is also necessary for SA-induced flowering to occur (Khurana and Maheshwari 1983). These observations thus reinforce the view presented earlier (Tanaka et al. 1979, Watanabe and Takimoto 1979, Khurana and Maheshwari 1983, see also Watanabe et al. 1981) that for induction of flowering in duckweeds, SA, in all probability, acts in a manner different from chelation.

It is also of interest to mention here that for induction of flowering, SA is now known to simulate the effect of EDDHA in a number of plants, e.g. *Pistia stratiotes* (Pieterse 1978), *Spirodela punctata* (Scharfetter et al. 1978), *Lemna gibba* G_3 (see Tanaka et al. 1979), *L. paucicostata* 151

(Watanabe and Takimoto 1979), *L. paucicostata* LP_6 (Khurana 1982) and *Wolffia microscopica* (see Khurana and Maheshwari 1983). With regard to this similarity in the action of EDDHA and SA on flowering, a view has been presented earlier (Tanaka et al. 1979) that EDDHA may be effective due to presence of the salicyl-like moiety in its structure and *not* merely to its chelating activity, as has been speculated earlier (Tanaka et al. 1979). Further work will be required to determine if the flower inducing activity of EDDHA for *S. polyrrhiza* SP_{20} is in fact due to its salicyl type component.

A few lines concerning the changes in anthocyanin content would be in order. Whether EDDHA, SA and BA, have any direct role in anthocyanin formation is not yet clear. However, an inverse relationship of anthocyanin content with growth was observed more consistently and one would infer from this that the increase in anthocyanin formation is triggerred due to stress. But, since no detailed work was undertaken, the usefulness of these observations is more to stimulate further work.

The financial support by the Department of Science and Technology, Government of India, is gratefully acknowledged.

References

Bonner, J. and P. S. Devirian (1939) Growth factor requirements of four species of isolated roots. *Amer. J. Bot.* 26: 661–665.

Clark, N. A. (1925) The rate of reproduction of *Lemna major* as a function of intensity and duration of light. *J. Phys. Chem.* 29: 935–951.

Gupta, S. and S. C. Maheshwari (1970) Growth and flowering of *Lemna paucicostata*. I. General aspects and role of chelating agents in flowering. *Plant Cell Physiol.* 11: 83–95.

Heller, R. (1953) Recherches sur la nutrition minerale des tissus végétaux cultivés in vitro. *Ann. Sci. Natur. Bot. Biol. Vég.* 14: 1–223.

Khurana, J. P. (1982) In vitro control of flowering in duckweeds. *Ph.D. Thesis*, University of Delhi, Delhi.

Khurana, J. P. and S. C. Maheshwari (1980) Some effects of salicylic acid on growth and flowering in *Spirodela polyrrhiza* SP_{20}. *Plant Cell Physiol.* 21: 923–927.

Khurana, J. P. and S. C. Maheshwari (1983) Floral induction in *Wolffia microscopica* by salicylic acid and related compounds under non-inductive long days. *Plant Cell Physiol.* 24: 907–912.

Lacor, M. A. M. (1968) Flowering of *Spirodela polyrrhiza* (L) Schleiden. *Acta Bot. Neerl.* 17: 351–359.

Pieterse, A. H. (1978) Experimental control of flowering in *Pistia stratiotes* L. *Plant Cell Physiol.* 19: 1091–1093.

Pieterse, A. H., P. R. Bhalla and P. S. Sabharwal (1970) Investigations on the effects of metal ions and chelating agents on growth and flowering of *Lemna gibba*. *Plant Cell Physiol.* 11: 879–889.

Scharfetter, E., Th. Rottenburg and R. Kandeler (1978) Die Wirkung von EDDHA und salicylsäure auf Blütenbildung und vegetative Entwicklung von *Spirodela punctata*. *Z. Pflanzenphysiol.* 87: 445–454.

Seth, P. N., R. Venkataraman and S. C. Maheshwari (1970) Studies on the growth and flowering of a short-day plant, *Wolffia microscopica*. II. Role of metal ions and chelates. *Planta* 90: 349–359.

Smith, J. H. C. and A. Benitez (1955) Chlorophylls: Analysis in plant materials. *In* Modern Methods of Plant Analysis, Vol. IV. Edited by K. Paech and M. V. Tracey. pp. 142–195. Springer-Verlag, Berlin.

Tanaka, O., C. F. Cleland and W. S. Hillman (1979) Inhibition of flowering in long-day plant *Lemna gibba* G_3 by Hutner's medium and its reversal by salicylic acid. *Plant Cell Physiol.* 20: 839–846.

Watanabe, K., T. Fujita and A. Takimoto (1981) Relationship between structure and flowering inducing activity of benzoic acid derivatives in *Lemna paucicostata* 151. *Plant Cell Physiol.* 22: 1469–1480.

Watanabe, K. and A. Takimoto (1979) Flower-inducing effects of benzoic acid and some related compounds in *Lemna paucicostata* 151. *Plant Cell Physiol.* 20: 847–850.

Wolek, J. (1974) Experimental control of flowering in *Spirodela polyrrhiza* (L.) Schleid., strain 7401—a preliminary report. *Ber. Geobot. Inst. ETH Stiftung Rübel* 42: 163–170.

(Received January 14, 1986; Accepted May 10, 1986)