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Involvement of ethylene in the effect of EDDHA on flowering and vegetative development in *Spirodela punctata*

Die Bedeutung des Aethylens für die EDDHA-Wirkung auf Blütenbildung und vegetative Entwicklung von *Spirodela punctata*

by

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1. INTRODUCTION

Salicylic acid (SA) and EDDHA (ethylenediamine-di-o-hydroxyphenylacetic acid) belong to a group of substances, which promote flowering in long-day dependent species as well as in short-day dependent species of Lemnaceae (see KANDELER 1984, 1985). In Spirodela punctata O 5, a quantitative long-day plant, not only flower induction is influenced by EDDHA or SA, but also several further developmental processes (SCHARFETTER et al. 1978). Interestingly, the effects of EDDHA on flowering, root length, frond size, development of air chambers and papillae, can be diminished

or canceled by supply of 10^{-6}M x-naphthaleneacetic acid (NAA). This result has led to the assumption that EDDHA and SA are effective through a lowering of the endogenous auxin level (SCHARFETTER et al. 1978). In fact, EPSTEIN and BENTAL (1985), working with Lemna gibba G3, have shown recently that SA indeed has no influence on the total indoleacetic acid (IAA) content of plants, but lowers the portion of IAA, which is in a free form, from 80% to 40%.

In Lemna gibba G3 the effects of EDDHA and SA on the development of air spaces are very pronounced leading to the gibbosity of fronds. Ethrel (2-chloroethane phosphonic acid), an ethylene-releasing substance, influences gibbosity in L. gibba in a similar way as EDDHA and SA (PIETERSE 1976). This fact and the preliminary report of ELZENGA et al. (1980) that EDDHA increases the ethylene evolution in L. gibba G3, could be a hint that some of the EDDHA and SA effects on developmental processes come about not only by a lowering of endogenous auxin levels, but also by an increase in ethylene production.

A simultaneous decrease in free auxins and increase in ethylene formation would be, however, in contrast to what is known from other plants. In several cases a stimulation of ethylene evolution has been observed after an increase (not decrease) of auxin levels (ABELES 1973, LIEBERMAN 1979). Having this discrepancy in mind, we investigated the involvement of ethylene in the effect of EDDHA in Spirodela punctata, working with the ethylene-releasing substance ethrel and the ethylene precursor 1-aminocyclopropane-1-carboxytic acid (ACC) as well as the inhibitors of ethylene synthesis aminoethoxyrinylglycine (AVG), aminooxyacetic acid (AOA) and Co^{2+} (see YANG and HOFFMAN 1984). To clarify the specifity of hormonal effects in S. punctata, experiments with other hormones (kinetin, KI; benzyladenine, BA; gibberellin A_3, GA_3; abscisic acid, ABA) and the auxin antagonist p-phlorophenoxyisobutyric acid (PCIB) were also included.

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2. MATERIAL AND METHODS

The clone 0 5 of Spirodela punctata (G.F.W. MEYER) THOMPSON is held in axenic culture in our laboratory since 1975. The starting material for the clone was collected by J. KOHLMEYER in Newport, North Carolina, USA. The experimental material was precultivated and cultivated under long-day conditions (16 hours per day light from Osram-HQIL lamps. Light intensity 4.5 K Lux). Temperature was 26 ± 0.5° C during light, and 22 ± 0.5° C during dark phase. During preculture plants were grown in autoclaved PIRSON-SEIDEL medium (1950) with iron as Fe (III)-EDTA (ethylenediaminetetraacetic acid). The medium for experimental cultures was sterile-filtered and contained Fe (III)-EDTA (experiments of Table 1), or $FeSO_4$, $MnCl_2$, and in addition 1.95×10^{-5} M EDDHA (chelating iron as well as manganese) (Experiments of Tables 2-4). Also KI, GA_3 (Serva), BA, ABA (Sigma), PCIB (Apin Chemicals, Cardiff, Gt.Britain), ethrel (C.F. Spiess a. son, Kleinkarlbach, FRG), ACC (Calbiochem), AVG (Fluka), and AOA (Sigma) were added to the nutrient medium before sterile- filtration. In the case of PCIB experiments 0.25% dymethyl sulfoxide (DMSO) was added to all experimental groups. Each experimental group consisted of three replicates (two replicates only in some cases). Duration of the experiments was 14 days. All experiments were carried out at least twice.

For quantitative evaluation of flowering all visible fronds of a culture were examined for flowers under a dissecting microscope and the percentage of flowering fronds were determined after substraction of the 3-10 inoculation fronds. At the same time the percentage of one-rooted fronds was determined (S. punctata normally possesses 2-4 roots per frond). The growth rate was calculated with the formula of LANDOLT (1957). The items for root length are maximum values within one culture. All other characteristics were estimated with a relative gradation scale.

3. RESULTS AND DISCUSSION

The assumption that EDDHA acts on developmental processes in S. punctata through a lowering of the physiologically active auxin level, was tested in experiments with the anti-auxin PCIB. The results are summarized in Table 1 in a simplified form (evaluation of the original data was carried out in the same way as in experiments shown in Tables 2 and 3). At 10^{-5}M PCIB root length is inhibited; air chambers (on the lower side of fronds) and papillae (on the upper side of fronds) are promoted weakly. At $5x10^{-5}$M PCIB several characteristics are influenced similarly as after supply of EDDHA: The percentage of one-rooted fronds, the size of frond colonies (number of fronds hanging together), the visibility of air chambers, and the chlorophyll content increase, whereas root length, growth rate, and frond size decrease. No other agent tested so far has the capacity to mimic the EDDHA effect to such a high degree as PCIB (Table 1). This may be a confirmation for the above made assumption that EDDHA affects growth and development - at least partially - by a lowering of the endogenous free auxin level. The results, however, must be taken with some caution, because $5x10^{-5}$M PCIB causes in addition a vitrification of fronds (23 to 83% of fronds can be affected depending on the experiment). Nevertheless, the cited effects were found not only in vitrified, but also in healthy fronds.

A further result which can be drawn from Table 1 is the fact that variation of the endogenous ethylene level by ACC or $CoSO_4$ is largely ineffective under the conditions used (PIRSON-SEIDEL medium with Fe (III)-EDTA). Measurements of ethylene with gas chromatography (FÄRBER, unpubl. results) have shown that in Lemnaceae exogenous ACC is used very rapidly to form ethylene. Cobaltous ions, on the other hand, are known to inhibit the last step of ethylene formation (YU and YANG 1979).

The conclusion that ethylene plays no role in the developmental processes of S. punctata, would be, however, premature. When the effect of ethylene-releasing substances and ethylene-formation blockers is examined in the presence of EDDHA, then distinct effects on all the investigated characteristics can be stated. Table 2 presents the original data of an experiment with AVG. As one can see, most of the EDDHA effects are abolished or diminished by 10^{-8}M AVG. Largely the same results can be obtained with AOA as another inhibitor of ethylene formation (Table 4).

Table 1. Effect of EDDHA and several growth regulators on developmental processes in S. punctata. Control plants were cultivated in Pirson-Seidel medium with Fe(III) EDTA.

Tab. 1. Die Wirkung von EDDHA und einer Reihe von Wachstumsregulatoren auf die Entwicklungsprozesse von S. punctata. Die Kontrollpflanzen wurden in Pirson-Seidel-Lösung mit Fe(III) EDTA kultiviert.

For comparison experimental results are given in a simplified form:
Für einen besseren Vergleich sind die experimentellen Ergebnisse in einer vereinfachten Form wiedergegeben:
(+), +, ++ = weak, distinct or strong promotion, respectively
= schwache, deutliche, beziehungsweise starke Förderung.
(-), -, -- = weak, distinct, or strong inhibition, respectively
= schwache, deutliche, beziehungsweise starke Förderung
0 = kein Effekt - no effect.
Differing results from successive experiments are given separately (before and after a comma)
Unterschiedliche Ergebnisse in nacheinander ausgeführten Versuchen sind durch ein Komma getrennt.
x = iron added as $FeSO_4$, xx = with DMSO 0.15%

	EDDHA^{x}	PCIB^{xx}		ACC	Co^{2+}	KI	BA	GA_3	ABA
	1.95×10^{-5}M	10^{-5}M	5×10^{-5}M	10^{-7}M	10^{-5}M	10^{-7}M	10^{-7}M	10^{-7}M	10^{-7}M
flowering %	++	0	0	0	0	0	0	0	0
one-rooted fronds %	++	0	+,++	0	0	0	0	0	0
root length (mm)	-,+	-	-,--	(-)	-	-	--	--	-
growth rate	-	0	-	0	0,-	0	0	0	(-)
size of frond colonies	++	0	+	0	0	++	++	0	0
frond size	-	0	-	0	0	0	+	-	-
visible air chambers	+	(+)	+	0	0	+	0	0	0
papillae	+	(+)	0	0	0	0	0	0	0
chlorophyll content	+	0	+	0	0	-	-	(-)	0

Table 2. Effect of EDDHA and AVG on several developmental processes in S. punctata.
For percentage of flowering and one-rooted fronds, maximum root length and growth rate three counts per experimental group are given. For other characteristics estimated values are noted.

Tab. 2. Die Wirkung von EDDHA und AVG auf eine Reihe von Entwicklungsprozessen bei S. punctata.
Für die Blühprozente und den Prozentsatz einwurzeliger Sprosse, sowie für die maximale Wurzellänge und die Wachstumsrate sind jeweils drei Werte pro Versuchsgruppe angegeben. Für die anderen Merkmale sind geschätzte relative Stufenwerte angegeben.

EDDHA 1.95×10^{-5} M	-	+	+	+	+
AVG (M)	-	-	10^{-10}	10^{-9}	10^{-8}
flowering %	0, 0, 0	77,78,72	4,21,56	0, 0, 0	0, 0, 0
one-rooted fronds %	0, 0, 0	81,87,83	69,84,80	64,62,48	48,47, 45
root length (mm)	16, 16, 14	12,12,12	14,14,12	16,16,18	14,16, 18
growth rate	125,129,123	82,89,86	88,83,90	96,90,99	97,98,101
size of frond colonies	+	+++	++	+	+
frond size	+++	++	++	++	++
visible air chambers	-	+++	+++	+++	++
papillae	-/+	+++	+++	++/+++	++/+++
chlorophyll content	+	++	++	++	++

Table 3. Effect of EDDHA and ACC on several developmental processes in S. punctata. (For further details see Table 2)

Tab. 3. Die Wirkung von EDDHA und ACC auf eine Reihe von Entwicklungsprozessen bei S. punctata. (Nähere Erläuterungen s. Tab. 2).

EDDHA 1.95×10^{-5} M	-	+	+	+	+
ACC (M)	-	-	10^{-9}	10^{-8}	10^{-7}
flowering %	0, 0, 0	78,73,80	44,52,43	15,25, 6	1, 2, 0
one-rooted fronds %	0, 0, 0	84,82,82	81,83,80	78,82,77	54,72,61
root length (mm)	12, 12, 14	12,12,12	16,14,16	16,16,16	22,22,20
growth rate	116,118,118	81,81,89	94,91,91	99,91,99	97,95,95
size of frond colonies	+	+++	+++	++	++
frond size	+++	++	++	++	++
visible air chambers	-	+++	+++	+++	+++
papillae	-	+++	+++	+++	+++
chlorophyll content	+	++	++	++	++

Table 4. Effect of ethylene-synthesis blockers (AVG, AOA, $CoCO_4$) and ethylene-releasing substances (ethrel, ACC) on developmental processes in S. punctata.
Control plants were cultivated in Pirson-Seidel medium with 1.95×10^{-5} M EDDHA. Signification of signs see Table 1.

Tab. 4. Die Wirkung von Aethylen-Syntheseblockern (AVG, AOA, $CoSO_4$) und Aethylen-abgebenden Substanzen (Ethrel, ACC) auf Entwicklungsprozesse bei S. punctata. Die Kontrollpflanzen wurden in Pirson-Seidel-Lösung mit 1.95×10^{-5} M EDDHA kultiviert.
(Zeichenerklärungen s. Tab. 1).

	AVG 10^{-8} M	AOA 10^{-11} M	AOA 10^{-8} M	$CoSO_4$ 10^{-5} M	Ethrel 10^{-3} %	ACC 10^{-7} M
flowering %	--	--	--	--	--	--
one-rooted fronds %	-	-	--	0	-	-
root length (mm)	+	+	+	-	+	+
growth rate	+	+	+,-	0	-	+
size of frond colonies	--	-	-	--	-	-
frond size	0	0	+	0	0	0
visible air chambers	-	-	--	0	0	0
papillae	-	-	--	0,-	+	0
chlorophyll content	0	0	-	-	0	0

The results from Co^{2+} experiments show a lot of deviations in comparison to AVG and AOA (Table 4), but also cobaltous ions influence five characters in a specific way. That Co^{2+} has not the same 'operation pattern' as AVG and AOA may be caused by the fact that Co^{2+} acts on development not only through inhibition of ethylene formation, but also through inhibition of Ca^{2+} membrane transport (GOODWIN et al. 1983, GROTHA 1983, WAYNE and HEPLER 1984). Surprisingly, the ethylene-releasing substances ACC and ethrel influence 4(-5) characteristics in the same direction as AVG and AOA (Tables 3 and 4), i.e., are reverting the effect of EDDHA. Only the development of papillae is promoted further by ethrel beyond the promotion by EDDHA.

Two conclusions may be drawn from the cited results. Firstly, EDDHA changes the sensitivity to endogenous ethylene. Agents which modify the endogenous ethylene production, are effective on developmental processes of S. punctata only in the case that EDDHA is supplied to the plants. Secondly, there are some processes as flowering, determination of root number, root growth, and colony growth, which need a certain intermediate ethylene level. A decrease (by AVG and AOA) as well as an increase

(by ACC and ethrel) in endogenous ethylene removes the intermediate state of ethylene presence in the tissue and prevents - on the other hand - the realization of certain EDDHA effects.

The change in ethylene sensitivity can be made understandable by the assumption that a certain balance of other hormones is needed as a precondition for the action of ethylene. As stated above, EDDHA seems to decrease the endogenous free auxin. A second hormonal change induced by EDDHA possibly could be an increase in cytokinins. A hint for such a hypothesis is the fact that cytokinins are the only agents (besides PCIB) which can repeat a positive effect of EDDHA. KI and BA increase the size of frond colonies even more than EDDHA (Table 1). Furthermore, an increase of cytokinins simultaneously with the decrease of auxins would make understandable that an intermediate ethylene level is maintained after EDDHA supply. In other plants auxins and cytokinins both have a positive influence on ethylene formation (LIEBERMAN 1979).

The assumption that certain developmental processes are induced or inhibited by ethylene within a narrow range of concentrations only, can be corroborated for the induction of flowering. In the long-day plant Lemna gibba G1 flowering is promoted by 10^{-8}M ACC, but inhibited by 10^{-7}-3x 10^{-7}M ACC. In the short-day plant Lemna aequinoctialis 6746 not 10^{-8} or 10^{-7}M ACC, but 10^{-6}M ACC has a flower-promoting effect under long-day conditions (SCHARFETTER et al. in preparation).

Summarizing the results it may be said that for realization of EDDHA effects on development a certain level of ethylene has to be present as a necessary but not sufficient condition.

SUMMARY

Addition of the heavy-metal chelating substance EDDHA to the nutrient solution influences several developmental processes in Spirodela punctata, and some of these effects are diminished or canceled by the auxin NAA (SCHARFETTER et al. 1978).

In the present paper we show that the anti-auxin PCIB can mimic the EDDHA effects to a high degree. Ethylene-formation inhibitors (AVG, AOA, Co^{2+}) and ethylene-releasing substances (ACC, ethrel) are effective in the presence, but not in the absence of EDDHA. The EDDHA effects on flowering, root number, root length, and size of frond colonies are diminished or abolished by AVG and AOA as well as by ACC and ethrel.

The results are interpreted by the assumption that EDDHA decreases the endogenous free auxin level (and - possibly - increases the endogenous cytokinin level) in this way changing the sensitivity to endogenous

ethylene. Flowering and the other three above-named developmental processes seem to need a certain intermediate ethylene level, which can be suppressed by AVG and AOA or exceeded by ACC and ethrel.

ZUSAMMENFASSUNG

Die Zugabe des Schwermetall-Komplexbildners EDDHA zur Nährlösung beeinflusst eine Reihe von Entwicklungsprozessen bei Spirodela punctata. Einige dieser EDDHA-Effekte werden durch zusätzliche Gabe des Auxins NAA gemindert oder aufgehoben (SCHARFETTER et al. 1978).
In der vorliegenden Arbeit wird gezeigt, dass das Antiauxin PCIB einen Grossteil der EDDHA-Effekte imitieren kann. Die Blocker der Aethylenbildung AVG, AOA und Co^{2+}, sowie die Aethylen-freisetzenden Substanzen ACC und Ethrel sind wirksam in Gegenwart, jedoch nicht in Abwesenheit von EDDHA. Die EDDHA-Wirkungen auf Blütenbildung, Wurzelanzahl, Wurzellänge und Grösse der Sprosskolonien werden sowohl durch AVG und AOA, als auch durch ACC und Ethrel gemindert oder aufgehoben.
Die Ergebnisse werden dahingehend interpretiert, dass EDDHA den endogenen Spiegel des freien Auxins erniedrigt (und eventuell zusätzlich den endogenen Cytokinin-Spiegel erhöht), wodurch dann eine Empfindlichkeitsänderung gegenüber endogenem Aethylen herbeigeführt wird. Die Blütenbildung sowie die drei anderen obengenannten Entwicklungsprozesse scheinen auf einen mittleren Aethylen-Spiegel angewiesen zu sein, der sowohl (durch AVG und AOA) unterschritten, als auch (durch ACC und Ethrel) überschritten werden kann.

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