PHYTON
ANNALES REI BOTANICAE

VOL. 24. FASC. 1 PAG. 1—152 15. 2. 1984

Phyton (Austria)	Vol. 24	Fasc. 1	1—10	15. 2. 1984

The Excitatory Substance of the *Mimosaceae*

By

Wiebke Bielenberg, Hermann Esterbauer [1]), Marianne Hayn [1]) and Karl Umrath [2])

Received November 22, 1982

Key words: Excitatory substance, *Mimosa pudica, Neptunia plena*

Summary

Bielenberg W., Esterbauer H., Hayn M. & Umrath K. 1984. The excitatory substance of the *Mimosaceae*. — Phyton (Austria) 24 (1): 1—10. English with German summary.

In extracts of leaves of *Mimosa pudica* two activity maxima regarding the initiation of leaf movements were separated by chromatography on a sephadex column. The first maximum (E) had about 80%, the second (G) about 20% of the activity. β-glucosidase did not alter the activity of E, but destroied the activity of G.

Further experiments showed, that substance E was a carboxylic acid, probably aliphatic.

Substance G was a phenolic glycoside, which, in contrast to substance E, was not retained by an anionexchange filter.

Leaf extracts of *Neptunia plena*, another mimosaceous plant, had the same activity as extracts of *Mimosa pudica* regarding the initiation of leaf movements of *Mimosa*. On the sephadex column extracts of *Neptunia* had

[1]) Prof. Dr. Hermann Esterbauer, Dr. Marianne Hayn, Institut für Biochemie der Universität Graz, Schubertstraße 1, A-8010 Graz (Austria).

[2]) Prof. Dr. Karl Umrath, Institut für Zoologie der Universität Graz, Universitätsplatz 2, A-8010 Graz (Austria).

one activity maximum with the elution volume of the E activity of *Mimosa* extracts. By an anion exchange filter *Neptunia* extracts lost their whole activity, whereas *Mimosa* extracts retained some activity corresponding to the activity of the glycoside.

From these results it was concluded that the acid responsible for the E activity of a *Mimosa* extract is the excitatory substance of the *Mimosaceae*.

By experiments on *Mimosa pudica* sprouts with different liberating substances it was found, that the excitatory substance had one or more OH-groups and a carboxylic acid grooup in trans position.

Zusammenfassung

Bielenberg W., Esterbauer H., Hayn M. & Umrath K. 1984. Die Erregungssubstanz der Mimosaceen. — Phyton (Austria) 24 (1): 1—10. — Englisch mit deutscher Zusammenfassung.

In Extrakten aus Blättern von *Mimosa pudica* wurden durch Chromatographie an einer Sephadex-Säule zwei Wirkungsmaxima bezüglich der Auslösung von Blattbewegungen getrennt. Das erste Maximum (E) hatte etwa 80%, das zweite (G) etwa 20% der gesamten Wirksamkeit. β-Glucosidase veränderte die Wirksamkeit von E nicht, zerstörte aber die von G.

Weitere Versuche zeigten, daß Substanz E eine, wahrscheinlich aliphatische, Carboxylsäure war.

Substanz G war ein phenolisches Glycosid, das, im Gegensatz zu Substanz E, in einem Anionenaustauschfilter nicht zurückgehalten wurde.

Blattextrakte von *Neptunia plena*, einer anderen Mimosacee, hatten bei Auslösung von Blattbewegungen von *Mimosa pudica* dieselbe Wirksamkeit wie Blattextrakte von *Mimosa*. An der Sephadex Säule hatten *Neptunia*-Extrakte ein Wirksamkeitsmaximum mit dem Elutionsvolumen von E der *Mimosa*-Extrakte. Durch den Anionenaustauschfilter verloren *Neptunia*-Extrakte ihre ganze Wirksamkeit, während *Mimosa*-Extrakte eine gewisse Wirksamkeit behielten, die der Wirksamkeit des Glucosids entsprach.

Aus diesen Ergebnissen wurde geschlossen, daß die Säure, welche die E-Wirksamkeit eines *Mimosa*-Extraktes bedingt, die Erregungssubstanz der Mimosaceen ist.

Versuche an Sprossen von *Mimosa pudica* mit verschiedenen freisetzenden Substanzen zeigten, daß die Erregungssubstanz eine oder mehrere OH-Gruppen und eine Carboxylgruppe in trans Stellung hat.

Introduction

Different plant families have different excitatory substances (Umrath 1930, Umrath & Soltys 1936, Soltys, Umrath & Umrath 1938). Umrath 1930 found extracts of certain plants, including *Mimosa pudica*, exciting also plants of other families and he ascribed this to unspecific substances, occuring besides the excitatory substance. Extracts of *Neptunia plena* have very little effect on plants of other families. Therefore Soltys & Umrath 1936 and Soltys, Umrath & Umrath 1938 extracted leaves of *Neptunia plena*. They found that the excitatory

substance of the *Mimosaceae* is an oxyacid, possibly containing nitrogen, but not being an aminoacid. By high purfication the substance got very instable. This instability was also noted by HESSE 1939 and by HESSE, BANERJEE & SCHILDKNECHT 1957. The suggestion of these autors, that the excitatory substance of Mimosa is a reductone is refuted by the observation, that Tillman's reagence and ferrichloride even in excess do not reduce the excitatory power of an extract of Mimosa leaves (UMRATH 1959). Also the oxidizing enzyme reported by HESSE, BANERJEE & SCHILDKNECHT 1957 to be in unboiled extracts could not be verified by UMRATH 1959.

SCHILDKNECHT 1978 claimed as leaf movement factor of *Mimosa* a glycoside, 2,5-dihydroxybenzoic acid (gentisinic acid) bound to a hexose and a pentose. The excitatory substances of most plant families seem to have a high water solubility and a poor lipoid solubility. It is therefore questionable if the extraction method of SCHILDKNECHT 1978 comprised the excitatory substance of *Mimosa*. It is not to be seen from his paper how effective the portions were, that he discarted in the way of his purification.

This in mind we attemped to purify the excitatory substance of *Mimosa* by column chromatography.

Supplementary we made experiments about the initiation of leaf movements of *Mimosa pudica* by chemical substances. This sort of release of movement can be interpreted by liberation of the excitatory substance in the plant. After UMRATH & THALER 1980 one can estimate certain details of the chemical structure of the excitatory substance if one knows what chemicals are most effective in liberating it.

Material and Methods

The test was performed on sprouts of *Mimosa pudica*. The plants were grown in a greenhouse without shading. Sprouts with 2 to 4 leaves were cut from the plants with a sharp scalpel. Under water at least one internode was cut away to avoid air bubbles in the vessels. Then the sprouts were fixed in supports, not in the bright sunlight, the cut ends in small dishes with about 5 ml of water. After reexpansion of the leaves the sprouts were ready for testing. The water in a dish was withdrawn with a pipette and replaced by the solution to be tested. Excitatory movements of the leaves were a sign of a positive test. The time after which they occured and the number of leaves reacting was an indication of the activity of the solution. The solution was diluted in further tests, till no reaction occured.

At first we extracted leaves of *Mimosa pudica* with 80% ethanol, as SCHILDKNECHT 1978 did. After evaporation of the ethanol and resolving in water the activity was much less as that of an extract

of leaves shortly boiled in water. Therefore we adopted the method of short boiling 3 g of pinnae of *M. pudica* in 15 ml of water. Occasionally we extracted pinae of *Neptunia plena* in the same way.

The extracts were chromatographed on a Sephadex LH 20 column of 2.5 cm diameter, filled to a height of 35 cm with methanol/water 92 : 8 (v : v) as mobile phase. The extract was purged through the column at a flow rate of approximately 1 ml/min.

For the biological test the methanol water effluent was evaporated at 40° C under reduced pressure and the residue was redissolved in water.

Aqueous solutions were passed through columns of polyamide (Woelm), 0,44 cm in diameter and 5 cm long, to retain phenols and through such columns of anion exchange resin (Amberlite CG 400 II).

Experiments on *Mimosa* shoots about chemically induced leaf movements interpreted by liberation of the excitatory substance were performed as described by Umrath & Thaler 1980.

Results

1. Preliminary separation and partial characterization of the active substances

Leaf extracts of *Mimosa* were seperated on a Sephadex column as described above. The effluent was fractioneted in 10 ml portions and each fraction or pooled fractions respectively were tested for activity in the biological assay. These experiments revealed, that the activity is eluted in two peaks differing in the elution volume and well separated by a distinct minimum. The first activity maximum (substance E) appeared with an elution volume of 129 ml, while the second (substance G) was eluted at 204 ml. The proportion of the activities was 80% for E and 20% for G. The recovery of the activity present in the original extract was about 70% as indicated by suming up the activities of all fractions of the column separation. This corresponds to earlier findings that the excitatory substance becomes unstable and looses activity upon extensive purification. (Soltys & Umrath 1936, Hesse 1939, Hesse, Banerjee & Schildknecht 1957).

Aqueous solutions of the active material recovered by these separations were stable up to several days at at 20° C and neutral pH.

Treatment with 2 mg/ml β-glucosidase for 2,5 hours at 50° C did not affect the activity of substance E, but completely abolished that of substance G. Therefore one can assume that the activity of substance E does not depend on a glycoside structure, while substance G most likely has a glycosidic bond necessary for ist activity.

In order to obtain more information on the chemical nature of substance E and G, aqueous solutions were passed through small

columns of polyamide and anion exchange resin. The former should retain phenols while the latter should retain substances containing the carboxyl grouping. These experiments revealed, that substance E is most likely a non-phenolic carboxylic acid, since it was completely retained by the anion exchanger column but not at all by the polyamide column. If the anion exchange column was washed with 10 ml of 1 M NaCl a small percentage (approximately 2%) of the activity applied to the column could be recovered in the effluent. It has to be said, however, that the high NaCl concentration depresses the response of *Mimosa* to the excitatory substance, thus the 1 M NaCl solution contained most likely 10% or more of the parent activity. If a 5 mM NaCl solution or a solution of 5 mM trans-cinnamic acid Na salt was used for the elution of the activity from the anion exchange column, 20% of the original activity was recovered. Lowering of the pH had no better results.

No further experiments to obtain a better recovery of substance E were carried out, since the anion exchange column experiment was primarily designed to proof the fact, that the substance E possess a carboxylic acid group.

The Sephadex LH 20 column fraction containing substance E was also separated by thin layer chromatography on silica gel with ethyl acetate/methanol/acetone/water 60; 15 : 10 : 10 (v : v). The fluorescence quench showed three spots at $R_f = 0{,}07$, 0,38 and 0,93. The latter was also detectable by its brownish color. The silicagel fractions corresponding to $R_f = 0{,}38$ and 0,93 were scraped off and extracted with water followed by water acetic acid 95 : 5. The water extract contained nearly no activity, while the dilute acid extract clearly showed an activity of the material eluted from the R_f area 0,38, but none from the R_f area 0,93. Spray the plate with the phenol reagent perchloride and ferric chloride (50 ml 35% $HClO_4$ + 1 ml 0,5 M $FeCl_3$) gave a positive reaction for the spot on $R_f = 0{,}93$ but a negative for the spot at $R_f = 0{,}38$.

A sample of E from the Sephadex column had an ultra-violet absorption maximum at 270 nm characteristic for an aromatic compound. This ultra-violet absorption disappeared by passing an aqueous solution of E through the small polyamide column. After thin layer chromatography and extraction with water acetic acid 9 : 1 the material absorbing at 270 nm was in the inactive fraction from the R_f area 0,93 and the active material of the R_f area 0,38 was completely free from any absorption at 270 nm.

All the above reported findings strongly suggest, that the excitatory substance E is a carboxylic acid and does not possess an aromatic structure.

As mentioned earlier, the activity of substance G, which accounts for about 20% of the total activity of the *Mimosa* extract, was destroyed by treatment with β-glucosidase. Furthermore it was completely retained by the polyamide column whereas it passed freely through the anion exchange column. One therefore can assume, that this substance is a glycoside, whose aglycon is a phenol but not a carboxylic acid.

The phenolic structure of substance G seems to be the reason for its high elution volume of 204 ml compared with the elution volume of 129 ml of substance E with a most probably aliphatic structure.

An extract of *N. plena* chromatographed on the Sephadex LH-20 column showed one activity maximum with the same elution volume as substance E of an extract of *M. pudica.*

We next studied *M. pudica* and *N. plena* extracts without using Sephadex chromatography. In the test on *M. pudica* the extracts of *Neptunia* had the same activity as those of *Mimosa.* Both extracts were run through small columns of anionexchange resin. Thereby the extracts of *N. plena* lost nearly their whole activity, whereas the extracts of *M. pudica* retained always a certain activity up to 10% of that of the original extract. It is reasonable to assume, that this activity is due to the substance G.

2. Liberation of the excitatory substance by chemical substances

Umrath & Thaler 1980 found in their experiments about the liberation of the excitatory substance and of auxin in *Lupinus albus* hypocotyls, that indolyl-3-acetic acid is best liberated by acids if the following criteria are fulfilled. If the acid groups of both substances are in contact the residual structures should harmonize in some extent, but OH-side groups should not be on corresponding places, optimal they should be abundant on one of the substances and absent on the other. In a subsequent paper on the liberation of xanthoxin it will be shown, that also the fitting together of the two substances in respect to cis- and trans-positions is of great importance. This paper will also prove once more the criteria postulated by Umrath & Thaler 1980 for the liberation.

In table 1 we give for 30 liberating substances the mM concentrations necessary to induce leaf movements in *Mimosa pudica.* The carboxylic acids were tested in solutions neutralized with $NaHCO_3$. Additionally we give figures indicating the liberating power of the substances for liberation of the excitatory substance of a fabaceous plant, *Lupinus albus.*

Lanoline-water-paste with the respective substance in 15, 75 or 450 mM concentration was unilaterally administrated to hypocotyls of

Table 1

Concentrations of certain substances (mM) needed for liberating the excitatory substances of *Mimosa*, and the approximately corresponding figures for eliciting bendings of *Lupinus* hypocotyls. Interpretation of the figures see the text

	Substance	*Mimosa*	*Lupinus*
1	benzoic acid	10	10
2	2-hydroxybenzoic acid (= salicylic acid)	20	5
3	2-acetoxy-benzoic acid (= aspirin)	20	20
4	2,5-dihydroxybezoic acid (= gentisinic acid)	50	10
5	3, 4, 5-trihydroxy-bezoic acid (= gallic acid)	100	100
6	phenyl-acetic acid	20	20
7	α-hydroxy-phenyl-acetic acid (= mandelic acid)	20	1
8	4-hydroxy-3-methoxy-mandelic acid	30	3
9	phenyl-propionic acid (= hydrocinnamic acid)	10	5
10	3, 4-hydroxy-phenyl-propionic acid (= hydrocaffeic acid)	100	100
11	phenyl-benzoic acid (= biphenyl-4-carbonic acid)	5	
12	phenoxy-acetic acid	25	10
13	2, 4-dichlor-phenoxy acetic acid	2	5
14	phenoxy-butyric acid	20	
15	phenoxy-benzoic acid	20	
16	trans-cinnamic acid	2	1
17	trans-2-hydroxy-cinnamic acid	5	30
18	trans-3-hydroxy-cinnamic acid	5	20
19	trans-4-hydroxy-cinnamic acid	10	5
20	cis-4-hydroxy-cinnamic acid	40	100
21	trans-2-methoxy-cinnamic acid	20	3
22	cis-2-methoxy-cinnamic acid	50	30
23	trans-3,4-dihydroxy-cinnamic acid (= caffeic acid)	20	30
24	trans-4-hydroxy-3-methoxy cinnamic acid (= ferulic acid)	20	30
25	histidine	100	5
26	1-methyl-histidine	50	1
27	indolyl-3-acetic acid	1	1
28	5-hydroxy-indolyl-3-acetic acid	10	100
29	indolyl-3-propionic acid	3	10
30	indolyl-3-butyric acid	3	

L. albus. The elicited bendings were noted one and two days later. The whole experiments were performed in the dark.

If a substance in 15 mM concentration in the paste induced a hight percentage of bendings to the side of the paste, it was designated as 1. The higher concentrations of the substances in the paste were needed for bendings to the side of the paste and the less was the

percentage of bendings to this side, the higher are the figures in the table. 100 means that even with a 450 mM paste the bendings were mostly away of the side of the paste. This procedure gives figures approximatively corresponding to these for *Mimosa*.

Conclusions

The acid (E) that we found is equivalent to the oxyacid purified by SOLTYS & UMRATH 1936 and by SOLTYS, UMRATH & UMRATH 1938. The very poor lipoid solubility found by SOLTYS & UMRATH 1936 corresponds to our opinion, that the substance is likely to be an aliphatic one.

The glycoside (G) that we found, should reasonably be equivalent to the glycoside described by SCHILDKNECHT 1978 from *Mimosa*. There is conformity that the aglycon is a phenol. According to SCHILDKNECHT 1978 the aglycon is an acid, but in our experiments the glycoside flowed freely through the anion exchange column. This difference remains to be elucidated.

An extract of the mimosaceous plant *Neptunia plena* contains a substance exciting *Mimosa pudica* and having on the Sephadex LH-20 column the same elution volume as substance E from an extract of *Mimosa pudica*, which has 80% of the activity of such an extract. This substance is a carboxylic acid, as it is retained completely in the anion exchange column. The remaining 20% of the activity of a *Mimosa* extract are due to the glycoside G.

UMRATH 1930 came to the opinion, that every family or subfamily of plants has an own excitatory substance, which can be extracted from every plant of this group. Whereas the excitatory substance excites only plants of the own group and has a physiological significance. there are in some plant extracts substances, which excite also plants of other families and therefore probably have nothing to do with the physiological process of excitation. The glycoside G may be such a substance and is likely to be simply an end product of the metabolism.

UMRATH & SOLTYS 1936 found by chemical experiments that the excitatory substance of the *Fabaceae* and that of the *Mimosaceae* are very similar, both being oxyacids, that of the *Fabaceae* probably having more OH-groups. Our liberating experiments are in full accordance with these results. Table 1, summarizing these experiments, shows a general parallelism between the mM concentrations of the tested substances needed for liberating the excitatory substance of *Mimosa* and corresponding figures for *Lupinus*, a fabaceous plant. The liberation is hindered by OH-groups on places of both substances which come in contact or even in neibourhood during the liberation. Therefore the OH-groups found by UMRATH & SOLTYS 1936 cause higher figures in

table 1 of liberating substances with OH-groups, especially if a substance has two or three OH-groups. Exemples are No 5 compared with 1—3, No 8 compared with 7, No 10 compared with 9, No 23 and 24 compared with 16 and No 28 compared with 27.

The hitherto reported demonstrates the accordance between the results of UMRATH & SOLTYS by chemical experiments and our findings by liberation experiments. But our experiments can beyond it give some indication where OH-groups are situeted on the excitatory substances. Comparing No 17 to 19 with one another and all with No 16 the following is to be seen. For *Mimosa* the figures of No 17 to 19 are augmented, but most that for 19, whereas for the fabaceous plant *Lupinus* the figures of No 17 to 19 are also augmented, but essentially more these for No 17 and 18. From these findings it can be concluded that the excitatory substance of the *Mimosaceae* has an OH-group on the seventh C-atom counted from the carboxylic C as the first and the excitatory substance of the Fabaceae has OH-groups on the fifth and on the sixth C-atom from the carboxylic acid C. In accordance with this the ratio of the concentrations needed for liberation of indolyl-3-acetic acid to 5-hydroxy-indolyl-3-acetic acid, with the OH-group on the sixth C-atom from the carboxylic acid C, is 1 : 10 in the *Mimosaceae,* No 27 and 28 in table 1, and 1 : 1000 in the *Fabaceae,* as found by UMRATH & WATANABE 1983 in experiments on tendril coiling of *Pisum sativum* (*Fabaceae*). No 2, 7 and 8 have low figures for the *Fabaceae*, this means an OH-group on the third or on the second C-atom from the carboxyl-C provides a high liberating power. This may be taken as an indication, that the excitatory substance of the *Fabaceae* may have no OH-groups on these C-atoms. Also No 25 and 26, histidine and 1-methyl-histidine, are much more effective on the *Fabaceae* as on the *Mimosaceae*. Here the NH_2-group on the second C-atom could be the cause. No 2, 3 and especially 4 have high figures, low liberating power, in the test on Mimosa. The reason may be the following. The 2-hydroxy on the benzol-ring is, according to the direction in that one counts along the ring, the third or the seventh C from the caboxylic C and the 5-hydroxy-group on the benzol-ring is the fourth or the sixth from the carboxyl C.

All trans-compounds in table 1 are more effective in liberating the excitatory substance of the *Mimosaceae* and that of the *Fabaceae* as the cis-compounds or as a hydrocompound without a double bound. Compare No 19 with 20, 21 with 22, 16 with 9 and 23 with 10. This indicates that both these subfamilies of the *Leguminosae* very likely havee carboxylic acid groups in trans-position. Hitherto this was not found for any other plant family investigated.

A comparision of No 1, 6, 9 and 11 shows no dependence of the liberating ability from the length of the side chain of the benzol-ring.

This makes it more likely that the excitatory substance of the *Mimosaceae* and that of the *Fabaceae* are aliphatic than aromatic. This is in accordance with the supposition on the chemical basis.

Lastly the high liberating power of indolyl-3-acetic acid, No 27 in table 1, may be understood after UMRATH & WATANABE 1983 in the following way. UMRATH, UMRATH & SOLTYS 1938 found an antagonism between the excitatory substance and indolyl-3-acetic acid. This may have physiological importance and brought about by natural selection chemical similarities of both substances, being the reason for the liberating power of indolyl-3-acetic acid.

References

HESSE G. 1939. Über die Natur der Erregungssubstanz von *Mimosa pudica* L. — Biochem. Zeitschr. 303: 152—163.

—, BANERJEE E. & SCHILDKNECHT H. 1957. Die Reizbewegungen der Mimosoideen und ihre Hormone. — Experientia 13: 13—19.

SCHILDKNECHT H. 1978. Über die Chemie der Sinnpflanze *Mimosa pudica* L. — Sitz,-Ber. Heidelb. Akad. Wiss. Math.-naturwiss. Kl., Jg. 1978: 7—32.

SOLTYS A. & UMRATH K. 1936. Über die Erregungssubstanz der Mimosoideen. — Biochem. Zeitschr. 284: 247—255.

— — & UMRATH CH. 1938. Über Erregungssubstanz, Wuchsstoff und Wachstum. — Protoplasma (Berl.) 31: 454—480.

UMRATH K. 1930. Über Erregungssubstanzen. — Jb. wiss Bot. 73: 705—719.

— 1959. Der Erregungsvorgang. — Hb. Pflanzenphysiologie. Encyclopedia of Plant Physiology (Ed.: RUHLAND W.) 17/1: 24—110. Springer Verlag, Berlin—Göttingen—Heidelberg.

— & SOLTYS A. 1936. Über die Erregungssubstanz der Papilionaceen und ihre zellteilungsauslösende Wirkung. — Jb. wiss. Bot. 84: 276—289.

— & THALER I. 1980. Auslösung von Blattbewegungen bei *Mimosa* und von Krümmungen von *Lupinus*-Hypokotylen, gedeutet durch Freisetzung von Erregungssubstanz und Auxin. — Phyton (Austria) 20: 333—348.

— & WATANABE Sh. 1983. The hormonal basis of tendril coiling. — Phyton (Austria) 23 (1): 307—312.