PRESENCE OF GIBBERELLIN-LIKE SUBSTANCES AND THEIR POSSIBLE ROLE IN AUXIN BIOPRODUCTION IN ROOT NODULES AND ROOTS OF LUPINUS LUTEUS L.

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SUMMARY

Studies based on bioassay techniques showed that root nodules of *Lupinus luteus* L. contain substantially more methanol-extractable gibberellin-like substances than the roots do. Experiments done *in vitro* with sterile, cell-free enzyme preparations of nodule tissue revealed that the bioproduction of IAA from L-tryptophan is promoted by GA_3. These results are discussed in the light of current theories about action of gibberellins as related to auxin in plants.

1. INTRODUCTION

The only available data on the presence of gibberellin-like substances in leguminous root nodules and roots are those presented by RADLEY (1961), whose dwarf-pea bioassay results showed that ethanol extracts of root nodules of *Phaseolus vulgaris* and *Pisum sativum* contain substantially higher amounts of gibberellin-like substances than the root extracts. Because of the need for more information about this subject and in connection with the investigation on the auxin content of root nodules and roots of *Lupinus luteus* published in a previous paper (DULLAART 1967), a study on the contents of gibberellin-like compounds of root nodules and roots of this same plant species was undertaken after some preliminary work in this laboratory (VAN HAASTEREN, not published), emphasis being laid on quantitative comparison.

Several bioassay techniques were used, since it is known that the responses of biotests vary considerably with respect to the numerous gibberellins known to occur in higher plants.

In view of the results obtained by VALDOVINOS *et al.* (1967), VALDOVINOS & SASTRY (1968), LANTICAN & MUIR (1967), and MUIR & LANTICAN (1968), special attention has been paid to the possible influence of gibberellin (GA_3) on the production of IAA from tryptophan in incubation mixtures with sterile, cell-free enzyme preparation from root nodule tissue.

2. MATERIALS AND METHODS

Plant material

Lyophilized nodules, and roots minus the nodule zones were taken from lupine plants (*Lupinus luteus* L., "bittere gele lupine"), grown in the field for about 45 days.

Extraction:

The lyophilized tissue (usually in 10 g portions) was powdered with sand in a mortar, mixed with 100 ml absolute methanol, and kept at 0 °C for 24 hours. The mixture was then passed through Whatman No 1 filter paper; the filtrate was taken up in 100 ml 80% methanol and again allowed to stand at 0 °C for 24 hours. After a second filtration the filtrate was mixed with 100 ml 80% methanol, violently shaken several times and again filtered. The clear solutions were pooled and evaporated under reduced pressure at 50 °C until only an aqueous solution remained. This turbid solution was centrifuged for 20 min at about 2000 g. After the pH of the supernatant had been adjusted to 8.2 with KOH, extraction with ethyl acetate was performed three times. This ethyl acetate fraction (E_1) contains the basic gibberellin-like compounds.

The residual aqueous fraction was acidified to pH 2.5 with H_2SO_4 and extracted three times with ethyl acetate. This ethyl acetate fraction (E_2) contains the acid gibberellin-like substances.

The residual aqueous fraction was then mixed with H_2SO_4 – the end concentration of H_2SO_4 being 0.4 N – and held at 60 °C for 1 hour in order to liberate the bound gibberellin-like substances. Then the pH was reduced to 2.5, followed by extraction with ethyl acetate. This ethyl acetate fraction contains the bound gibberellin-like compounds (E_3).

The three ethyl acetate fractions were dried with anhydrous Na_2SO_4 and evaporated under reduced pressure at 60 °C until dry. The residues were taken up in a small volume of 96% ethanol.

It should be mentioned that the extraction of the nodule material was rather difficult and laborious, and the resulting fractions were rather dirty.

Chromatography

For further purification and partition, paper chromatography was applied. Before use the paper was washed with 96% ethanol. The solvent was isopropanol: ammonia (sp. gr. 0.96): water (10: 1:1). The fraction samples were spotted in bands about 3 cm long and after equilibration for 60 min. allowed to run for 17 hours. After drying with hot air, one of the chromatograms was sprayed with a solution of 5% H_2SO_4 in ethanol and inspected in UV light for fluorescent spots.

Bioassay techniques

Lettuce hypocotyl assay: The technique used was essentially according to FRANKLAND & WAREING (1960). The seed was *Lactuca sativa* cv. "Meikoningin".

Barley endosperm test: This test was performed according to JONES & VARNER (1967) with *Hordeum vulgare* cv. "Himalaya" seeds.

Dwarf pea epicotyl test: The technique according to PHILLIPS & JONES (1964) was performed with *Pisum sativum* cv. "Meteoor" seeds.

The chromatograms for bioassay were cut into pieces of one Rf unit each and eluted in distilled water or ethanol, the latter solvent being evaporated before dissolving the residues in the test solution. In each bioassay a number of GA_3

concentrations ranging from 0 to 1.0 μg/ml were measured for growth activity to construct a calibration curve. Therefore the growth activities found in the eluates of the chromatograms are expressed in amounts of GA_3.

Loss

In an attempt to evaluate the extent of the loss, known amounts of GA_3 were treated in the same way. The recovered amounts differed considerably, and since nothing is known about losses of other gibberellin-like substances during extraction and chromatography, the presented data were not corrected for losses and must therefore be considered as being of relative value.

Effect of GA_3 on bioproduction of IAA from L-tryptophan *in vitro*

The methods used for preparing the sterile, cell-free nodule enzyme extract, incubation, sterility check, IAA extraction, chromatographic purification and separation, spectrofluorometric identification, and quantitative estimation, have been described elsewhere (DULLAART 1970). The incubation mixtures contained the following constituents per 10 ml (final concentrations) in 0.05 M phosphate buffer (pH 6.5): 10 mg L-tryptophan, 7.2 mg α-ketoglutaric acid, 1 mg pyridoxal phosphate, and 1.0 mg GA_3; the last three compounds were added facultatively. To 9.5 ml substrate solution, 0.5 ml of dialyzed enzyme preparation was added, giving a protein content of about 5 mg per 10 ml incubation mixture. The entire incubation procedure was performed under strictly sterile conditions.

3. RESULTS

The lettuce hypocotyl assay gave the best responses, showing the presence of gibberellin-like substances in the extracts of root nodules and roots; the responses in the other two bioassays were very weak.

Fig. 1 shows representative results in the form of histograms of the gibberellin-like activities present in the chromatograms of the various fractions of the nodule and root extracts as demonstrated by the lettuce biotest. Evidently the E_2 fraction contains the bulk of the gibberellin-like substances. The responses obtained in both the other bioassays were too weak to yield reliable histogram patterns.

Table 1 shows the quantitative results concerning the activities present in the chromatograms of the different fractions, estimated by means of the different bioassays, and expressed as amounts of GA_3.

The quantitative differences between the results obtained in the lettuce hypocotyl test on the one hand and the other two assays on the other, are very striking. Evidently the nodule tissue contains considerably more gibberellin-like substances than the roots.

Judging from the fluorescence colour after treatment of chromatograms with the H_2SO_4 reagent and the large quantitative differences demonstrated in the bioassays, it seems highly improbable that GA_3 is one of the gibberellin-like

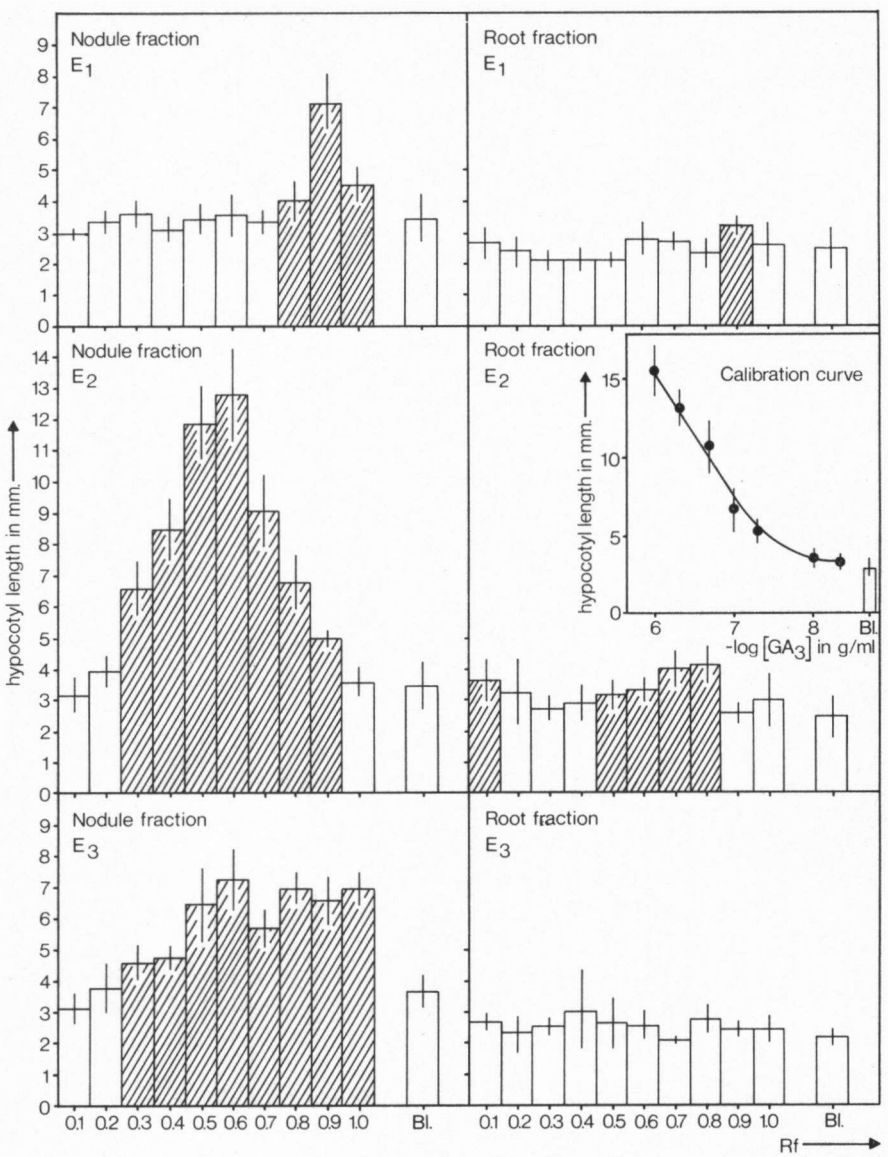

Fig. 1. Presence of gibberellin-like substances in the different fractions of the nodule and root extracts, demonstrated in the lettuce bioassay.
Standard deviation is given as vertical lines. Significant growth responses ($\alpha = 0.05$) are indicated by hatching.

Table 1. Gibberellin-like activities in different fractions of nodule and root extracts, expressed as μg GA_3 equivalent per kg fresh weight.

Extraction number		Bioassay	μg GA_3 equivalent per kg fresh weight in the different fractions		
			E_1	E_2	E_3
Nodules	1	lettuce hypocotyl	—	2100	460
	2	lettuce hypocotyl	340	3340	600
	2	barley endosperm	—	24	—
	3	lettuce hypocotyl	100	1100	—
	3	barley endosperm	—	5,4	—
	4	lettuce hypocotyl	—	420	—
	5	lettuce hypocotyl	—	1420	—
	5	barley endosperm	—	20	—
	5	dwarf pea epicotyl	—	2	—
roots	6	lettuce hypocotyl	8	56	0
	7	lettuce hypocotyl	—	24	—
	7	dwarf pea epicotyl	—	0	—

substances present in these tissues. However, since GA_3 was the only gibberellin available as pure chemical and since it was thought desirable to investigate the possible influence of gibberellins on the bioproduction of IAA from L-tryptophan in nodule tissue, GA_3 was applied in these experiments. The results are summarized in *table 2.*

From these data some conclusions may be drawn. The high stimulation of the IAA production in the presence of α-ketoglutaric acid and pyridoxal phosphate indicates that a transamination reaction is involved in this reaction sequence, indolepyruvic acid being an intermediary product. More evidence has been obtained in favour of this pathway in nodule tissue of Lupine (DULLAART 1970).

It may also be concluded that GA_3 stimulates the IAA bioproduction from

Table 2. Effect of GA_3 on production of IAA from L-tryptophan in incubation mixtures with dialyzed nodule-enzyme preparation.

Enzyme		Substrate + additives				Product
dialyzed	boiled	L-tryptophan	α-ketoglutaric acid	pyridoxal phosphate	GA_3	IAA (μg)
+	−	+	−	−	−	2.0±0.4
+	−	+	−	+	−	0.5
+	−	+	+	−	−	12.3±1.3
+	−	+	+	+	−	22.5±2.5
+	−	+	+	−	+	33.8±1.3
+	−	+	+	−	+	25.8±1.8
+	−	−	+	+	−	0.3±0.2
+	+	+	+	+	−	0.4±0.1
No enzyme		+	+	+	−	0.2±0.0

L-tryptophan via this pathway, since it seems capable of replacing pyridoxal phosphate as a transaminase cofactor. It must be stressed that thin layer chromatography of the extracts of these incubation mixtures showed no new bioproduction pathway of IAA to be induced by GA_3.

4. DISCUSSION

The results of this study show that root nodules of lupine contain substantially higher amounts of gibberellin-like substances than the roots. These findings may be considered to confirm the results of RADLEY (1961). Although the use of the fresh weight as reference measure might be considered questionable, it is very difficult to find a better one. The protein and the nitrogen contents, for instance, are not appropriate, because the nodule tissue is filled with the rhizobial symbiont and the protein and nitrogen levels are therefore very high, whereas the roots contain relatively much smaller amounts of protein and nitrogen, since they consist largely of woody transport tissue. But the difference between the contents of gibberellin-like compounds of nodule tissue and roots seem too high to be attributed to the unreliability of the fresh weight as reference measure.

The considerable differences between the responses in the different bioassays are surprising, but they might be ascribed to the difference between the sensitivities of the biotests to the gibberellin-like substances isolated from these tissues.

The stimulatory effect of GA_3 on IAA bioproduction from L-tryptophan in incubation mixtures with nodule enzyme extracts is very interesting in relation to the higher content of gibberellin-like substances of the nodules. It is conceivable that the higher IAA content of the nodules as compared to the roots (PATE 1958, DULLAART 1967) is due to stimulation of IAA bioproduction by gibberellins, which are present in higher amounts in the nodules. However, this supposition seems highly speculative. Moreover, DULLAART (1970) presented evidence suggesting that the IAA bioproduction in root nodules of lupine is not actually higher than the IAA production in young, actively growing roots, which means that differences between the IAA destruction activities of these tissues might be responsible for the difference of the IAA contents.

With respect to this point it may be mentioned that gibberellin has been reported to show "auxin-sparing" activity (ELEMA 1960, HOUSLEY & DEVERALL 1961). From the present results it is not possible to conclude with certainty whether the higher output of IAA was due to stimulation of the production and/or inhibition of the destruction of IAA known to occur in the mixtures during incubation (DULLAART 1970), either of which could be caused by GA_3. It would seem worth-while to pay special attention to the solution of this problem in future studies.

The demonstrated effect of GA_3 on the production of IAA *in vitro* is made even more interesting by the similar results obtained by VALDOVINOS *et al.* (1967), VALDOVINOS & SASTRY (1968), LANTICAN & MUIR (1967), and MUIR & LANTICAN (1968) with systems prepared from other plant species. However, it does not

seem likely that this is the only mode of action of gibberellins in these tissues (BRIAN 1965).

The coincidence of the higher contents of auxin and gibberellin-like substances in the nodules as compared to the amounts of these compounds in the roots is intriguing. It would be premature to conclude that the auxin concentration increases as a result of the increase of the amounts of gibberellin-like substances. It seems more probable that the increases of both auxin and gibberellin-like compounds are the manifestations of metabolic changes induced by the rhizobial infection.

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