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SIMILAR EFFECTS OF INDOLE-3-ACETIC ACID, NON-STERILE CONDITIONS AND CELLULASE ON THE EXTRACTABILITITY OF COMPOUNDS OF LOW MOLECULAR WEIGHT FROM BEAN ROOT

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Abstract

From earlier experiments it was concluded that the amounts of indole compounds extractable with methanol from plant material are affected by non-sterile conditions not only via the direct bacterial synthesis of indole compounds, but indirectly too. In a study of the nature of the indirect effect, the indole compounds of roots treated with cellulase, treated with indole-3-acetic acid (IAA) or maintained under non-sterile conditions were chromatographed, measured quantitatively and compared with the results from untreated controls. It was found that, compared to the control, all three treatments increased the concentrations of tryptophan, IAA and metabolite of IAA in roots grown in the culture. The extractable radioactivity was measured after similar samples had been fed with labelled glycine, and essentially similar results were obtained: more activity could be extracted with 20% ethanol from treated and non-sterile root samples than from the untreated controls. The experiments by and large support the assumption that the amounts of extractable indole compounds and radioactive gylcine are increased by exogenous cellulase, IAA-induced cellulase and bacterial cellulase via their cell wall loosening effects.

Introduction

The IAA metabolism of higher plants is well known to be closely connected with the apiphytic bacteria, for the presence of the microorganisms can affect both the synthesis and the decomposition of IAA. Accordingly, it is justified to assume that the conditions of sterility influence the occurrence of metabolites of IAA and their quantities in various plant organs.

We earlier reported the presence, isolation and partial identification of one of the metabolites of IAA in bean root (KÖVES and SIROKMÁN, 1973). In the present work we attempted primarily to prove that this metabolite is indeed formed in the bean root, and is not of bacterial origin. It emerged from the experiments that the compound is also formed under sterile conditions, and is thus a plant product; under non-sterile conditions, however, the amount of the substance isolable is higher than in the sterile sample. It was further observed that not only this IAA metabolite, but all of the other indole compounds too were present in larger amounts in the non-sterile roots. These observations turned our attention to the fact that the non-sterile conditions also have an indirect effect on the pattern and concentrations of the indole compounds extractable with methanol.

One explanation of the indirect effect may be the cell-exposing action of the cell wall decomposing enzymes produced by the bacteria. If our assumption is correct, then IAA, the growth-stimulating action of which is connected with the enzymatic

loosening of the cell wall, must exert an effect analogous with those of the cell wall decomposing enzymes and non-sterile conditions on the extractability of the above-mentioned compounds. On this basis, experiments were carried out in which bean root segments grown in sterile or non-sterile cultures were treated with IAA, cellulase or IAA + cellulase, and extracted with methanol in a completely uniform manner; their indole compounds were then chromatographed, and the chromatograms were evaluated with quantitative indices. A study was made of whether the cell-exposing effect induced by the cellulase preparation or by IAA is a general one, and extends to other compounds too, by extracting and measuring the radioactivity incorporated in the form of ^{14}C-labelled glycine in root segments which had been maintained under sterile or non-sterile conditions, and treated with IAA or cellulase.

Materials and Methods

Root culture

Dwarf bean seeds treated with neomagnol and bromine water and sterilized were germinated for 6 days in a sterile Petri dish; 2 cm segments were then cut off the root tips and put into liquid White medium. The cultures were kept in tne dark at room temperature for 2 weeks. Depending on the treatment desired, the sterile roots were next transferred to a further sterile medium containing IAA or cellulase. The weight of root mass taken in each sample was 2 g. The enzyme preparation was "Onozuka" $\beta-1\rightarrow4$-glucanase (EC 3.2.1.4), which was added to the medium in a concentration of 1%. The IAA (Merck) concentration of the media was 10^{-4} M.

Extraction of indole compounds, their thin-layer-chromatography, and their evaluation by UV spectrophotometry

For a correct assessment of the experiments, it was absolutely essential that all the samples should be extracted under completely identical conditions. The root samples were always homogenized for exactly 5 min with a four-fold amount of methanol, and extracted for 24 hr, the methanol being exchanged on two occassions; the methanolic solutions were combined, cetnrifuged, extracted three times with petroleum ether by shakign for 3 min, and evaporated to dryness in vacuum at 40°. The thin-layer-chromatograms were prepared on silica gel on 20×20 cm plates with an isopropanol--ammonia-water (10:1:1) solvent, identical quantities being taken at every start-point. In addition to the extracts, authentic samples too were run; identifications were made on the basis of these authentic samples, EHRLICH, SALKOVSKI and PROHÁZKA colour reactions, UV fluorescence and UV absorption curves. Values of the extinction or transmission of the peak at 280 nm were used for quantitative estimations. UV curves were recorded in the range 200—300 nm with a SPECORD UV-VIS spectrophotometer. All experiments were carried out in triplicate.

Extraction and measurement of radioactivity taken up by the roots in the forms of ^{14}C-glycine

The roots were grown in culture in the previously-described manner. 2 g of the 14-day roots was transferred to 15 ml White medium containing 5 μCi glycine. After 18 hr the roots incubated at room temperature (24°) were put into an Erlenmeyer flask with a medium containing 10^{-4} M IAA, 1% cellulase or no additive. A sterile and a non-sterile variant were prepared from the latter sample. A further 24 hr later the ^{14}Cr-labelled substances were extracted with 20% ethanol, completely identical conditions being employed for every sample.

The absorbed and extractable radioactivity was measured in toluene solution with a liquid scintillation method, on a Nuclear Chicago ISOCAP—300 spectrometer. A quench correction was applied.

Experimental results

1. Study of indole compounds extractable from roots treated with cellulase and with IAA in a sterile culture

The following indole compounds, in order of increasing Rf values, could be extracted thin-layer-chromatographically after dissolution in methanol from bean roots grown in a sterile culture, identification being performed on the basis of UV absorption and fluorescence: (0) an unidentified indole compound, (1) indole-3--acetylaspartic acid, (2) tryptophan, (3) indole-3-acetic acid, (4) a partially identified IAA metabolite, (5) an unidentified indole compound, an IAA metabolite.

Of these compounds, 0, 2 and 5 can be found in well-detectable amounts in roots grown in media either containing or without IAA, while 1 and 3 occur only in media provided with IAA. Quantitative examinations were carried out on compounds 2, 3 and 4. It did not prove possible to elute compounds 0 and 1 in appropriate amounts or purities from the chromatograms. From the sizes of the spots in the chromatogram shown in Fig. 1 it is evident that all of the indole compounds are present

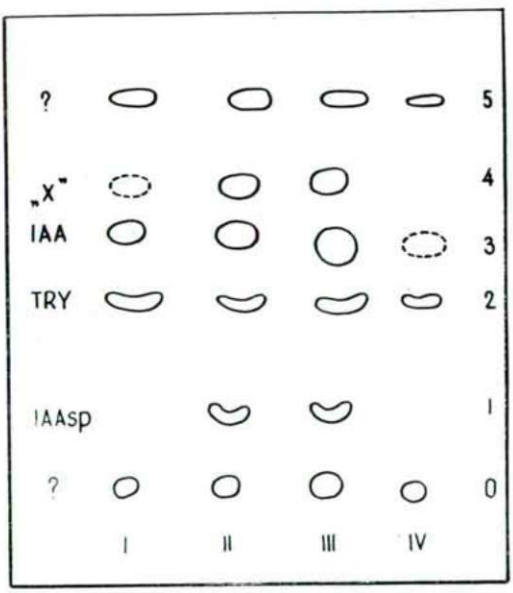

Fig. 1. Thin-layer-chromatograms of indole compounds extracted with methanol from bean roots grown in cultures under sterile conditions and treated in various ways. I: 1% cellulase; II: 10^{-4} M IAA; III: 1% cellulase + 10^{-4} M IAA; IV: untreated control. 0—5: see text.

in lower amounts in the untreated controls than in the other samples. The quantities of IAA (3) and the metabolite (4) increase in the following sequence in the variously--treated samples: untreated < cellulase treatment < IAA treatment

Comparison of the UV curves recorded on the eluates of the spots leads to a similar relative sequence (Figs. 2—4). The sequence relating to tryptophan is modified

in that the quantitative index (OD value) obtained on IAA treatment is somewhat less than in the case of cellulase treatment. Combined treatment with cellulase and IAA causes an approximately additive OD increase in the UV curve of tryptophan at 280 nm (Fig. 2).

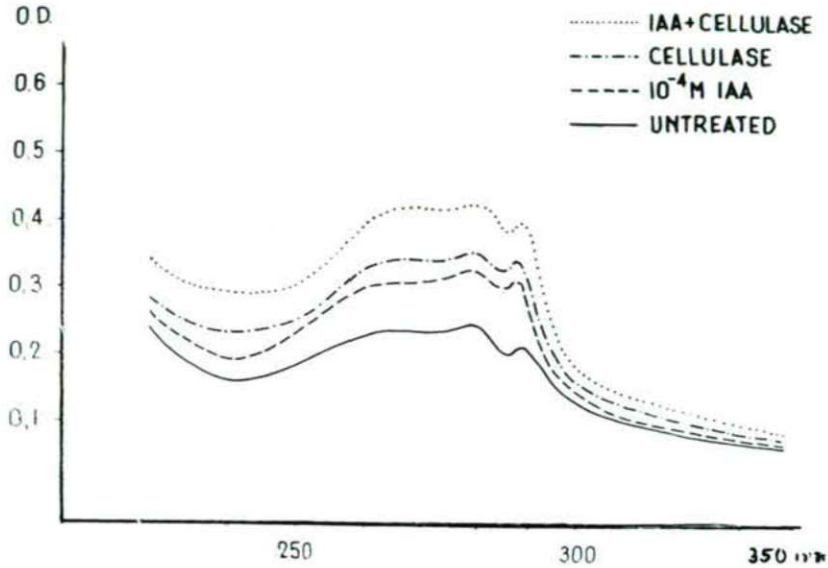

Fig. 2. Absorption curves in the range 230—350 nm of tryptophan (spot 2) eluted with methanol from the chromatogram in Fig. 1.

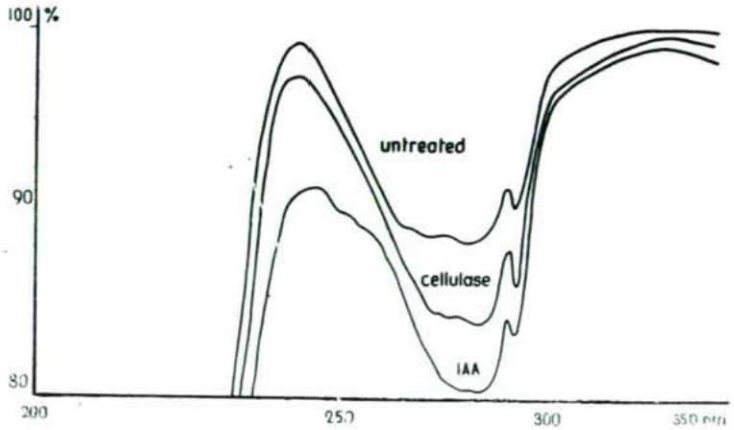

Fig. 3. Transmission curves in the range 230—350 nm of IAA (spot 3) eluted from the chromatogram in Fig. 1.

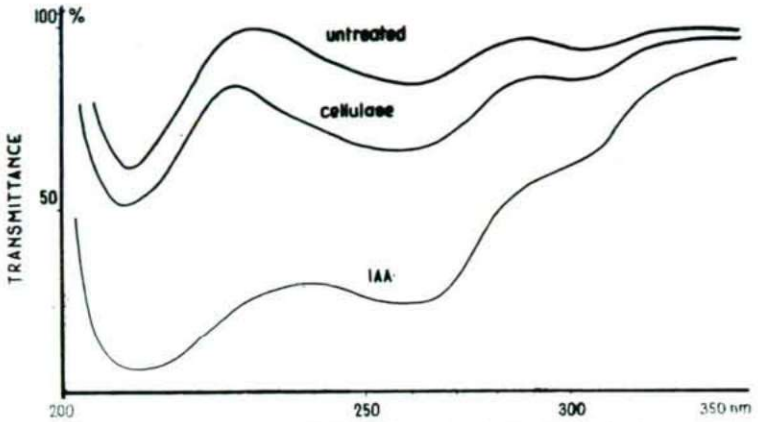

Fig. 4. Transmission curves in the range 230—350 nm of the IAA metabolite (spot 4) eluted with methanol from the chromatogram in Fig. 1.

2. Study of indole compounds extractable from roots grown in cultures under sterile and non-sterile conditions

Figure 5 presents the results of experiments comparing the effects of sterile conditions, non-sterile conditions, cellulase treatment and IAA treatment on the quantitative and qualitative relations regarding the indole compounds to be found in the roots.

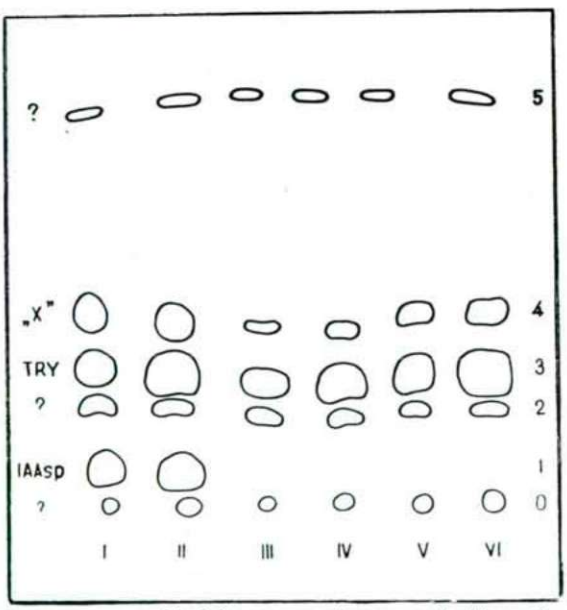

Fig. 5. Thin-layer-chromatograms of indole compounds extracted with methanol from bean roots grown in cultures under sterile or non-sterile conditions and treated or not treated with IAA or cellulase. I—II: 10^{-4} M IAA; III—IV: untreated; V—VI: 1% cellulase; I, III, V: sterile samples; II, IV, VI: non-sterile samples.

The indole compounds in sterile and non-sterile samples do not differ from one another qualitatively (Fig. 5). In both samples one finds: (0) traces of a compound giving the EHRLICH reaction, wich remains on the start-point, (2) an unidentified indole compound, (3) tryptophan, and (4) the already-mentioned IAA metabolite, which corresponds to spot 4 of Fig. 1. IAA-Asp (1) can betdetected only in roots incubated with IAA. The fact that IAA was not detectable in this chromatogram is explained in that the incubation time was raised to 24 hr, because of the ensuring of the non-sterile conditions. This time is necessary for the multiplication of the bacteria to an appropriate extent, whereas the free IAA is totally transformed to IAA-Asp or other metabolites.

Estimation on the basis of spot size shows that the amounts of compounds present in the non-sterile samples are generally higher than in the sterile ones. This holds for all of the indole compounds examined: IAA-Asp, Try and the partially-identified IAA metabolite.

It must be mentioned that while the effects of the various treatments (IAA, cellulase) were constant in every repetition in sterile cultures, the effect of the non-sterile conditions could not be unambiguously reproduced in every case. Thus, in certain cases, one or other or even all of the indole compounds give a smaller spot and a lower extinction compared to that in the sterile sample. To explain this, we suggest that the bacteria entering the medium and multiplying there are not always the same, and probably possess different enzyme reserves. Among them, for instance, may be some which cleave the glucosidic linkage of the metabolite of glucoside type, and as a consequence the metabolite is eliminated from the extract and from the chromatogram.

3. Radioactivity extractable with ethanol from roots fed with ^{14}C-glycine, and treated with IAA and cellulase. Effect of non-sterile conditions on the measurable amount of radioactive glycine

Roots grown in the manner described in the preceding points in the experimental series were incubated with ^{14}C-glycine, and four samples were then transplanted into (1) sterile White medium, (2) non-sterile White medium, (3) White medium containing 1% cellulase, and (4) White medium containing 10^{-4} M IAA. The radioactivities extractable with 20% ethanol after a 16-hr incubation are shown in Table 1.

Table 1

Treatment	cpm/mg fresh root	% of sterile control
1. Sterile untreated	743	—
2. Non-sterile untreated	1773	238
3. 1% cellulase	1979	266
4. 10^{-4} M IAA	1037	139

The conclusions drawn from the activities measured on the samples are in agreement with what has been said above. The highest activity is to be found in roots treated with cellulase, and then, in a decreasing sequence, in the IAA-treated, the non-sterile untreated, and the sterile untreated samples. Although the extents of the effects of the various treatments differ from those obtained in connection with the changes in extractability of the indole compounds, there is agreement in so far as all three treatments increase the amount of substance extractable compared to the control.

Discussion

One of the essential factors in the connection of auxin and the elongation growth is the auxin-regulation of the functioning and synthesis of the enzymes influencing the properties of the cell wall. The action of IAA in stimulating pectin methylesterase has long been known. More recently, attention has focussed on the study of the roles of enzymes degrading or forming cellulose and hemicellulose; these make possible cell elongation by loosening of the cell wall and synthesis of the materials. There is much indirect and direct evidence that in vitro the auxins affect the functioning of practically all the cell wall decomposing enzymes (β-1,3 and β-1,6-glucanase, cellulase, pectin methylesterase, β-1,4-galactanase, pectinase) (TANIMOTO and MASUDA, 1968; MASUDA, 1968; DATKO and MACLACHLAN, 1968; LABAVITCH, 1974; HEYN, 1969; MASUDA and YAMAMOTO, 1970).

According to TANIMOTO and MASUDA (1968), of the cell wall decomposing enzymes β-1,3-glucanase stimulates the elongation of barley coleoptile segments to roughly the same extent as IAA itself, while SHIMODA and YANAGASHIMA (1971) have demonstrated that auxin-sensitive yeast strains too are enlarged on the action of β-1,3-glucanase. In addition, a further interesting result has been reported: glucanase can be inhibited with γ-gluconolactone, and this substance also inhibits IAA-induced growth. These experimental results prove directly that the auxin effect is mediated by the glucanases.

This is supported in another respect by the experiments of DAVIES (1974), who showed that the elongation caused by auxin in pea epicotyl is proportional to the cellulase activity. According to HEYN (1969) and MASUDA and YAMAMOTO (1970), IAA substantially increases the activity of β-1,3-glucanase. The bulk of the glucanases is bound to the wall, from which it can be partially released with detergents. In contrast with the above, the work of RUESINK (1969) indicates that the auxins influence only the extensibility of the cell wall, which does not lead unconditionally to growth.

In his examination of the question, CLELAND (1971) also takes into consideration those auxin-induced permeability changes, which may similarly be responsible for the favourable modification of the connections between the enzymes and their substrates. Hence, the action of auxin in the loosening of the cell wall is to increase the permeability of the plasmalemma for certain enzyme proteins (e.g. various glucanases), and these cause the loosening of the cell wall by penetrating into it (LAMPORT, 1970). This is also supported by the studies by COCKING (1972) on protoplast, which indicate that auxin acts on the plasmalemma.

From the differences in reaction of the protoplast and the intact cell, however, YANAGASHIMA and SHIMODA (1968) conclued that auxin acts directly on the cell wall.

Accordingly, the manner in which the auxin effect is manifested in the cell wall has not yet been clarified as regards its details, but it is beyond doubt that in many objects the auxin effect is mediated by the glucanases.

This fact leads to the paradoxical situation that if plant matter is treated with exogenous auxin, this facilitates the transfer of the endogenous compounds into the solvent in the extraction procedure by enhancing the functioning of the cell wall loosening enzymes; this creates the impression that the amounts of the substances in the cell increase.

Consequently, as regards its nature, treatment with auxin is accompanied by a similar result as exogenous cellulase treatment.

In our reported experiments this theoretically derivable parallelism was confirmed in practice too, as the microbial action is also accompanied by cellulase production, while the non-sterile conditions too exhibit a similarity with the auxin effect as regards the extractability of endogenous substances. In our experiments this was demonstrated for indole compounds and amino acids.

These facts have important consequences from both methodological and theoretical aspects. One of these is that, even under sterile conditions, quantitative estimations or determinations based on extraction procedures may be misleading if carried out on plants immersed in IAA solution. Caution is particularly called for if relatively low differences are obtained in quantitative comparisons of various samples. It should be noted that the bulk of our information relating to the mode of action of IAA was derived from such examinations. The other conclusion is that in "in vivo" investigations undre sterile and non-sterile conditions, if the endogenous compounds of the two samples are being determined, an excess in favour of the non-sterile sample can not be attributed unconditionally and in its entirely to direct bacterial production, but may also be a result of readier extractability accompanying changes in the properties of the cell wall.

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