The Role of Endogenous Gibberellin-like Substances and Inhibitors in the Growth of Pea Internodes*

MIRJANA NEŠKOVIĆ and T. SJAUS*

Institute of Botany, Faculty of Science and Institute for Biological Research, Beograd

Received March 8, 1973

Abstract. Third internodes or whole stems of 7-days old etiolated pea plants were extracted and the content of gibberellin-like substances and inhibitors has been determined. Extracts were found to contain four or five different gibberellin-like substances, some of which are chromatographically similar to GA3. The content of gibberellins has been high in young internodes and decreased along with the internodes elongation. Brief red light irradiation brings about quantitative changes in gibberellin content, depending also on the length of internodes. The extracts contain acidic and neutral inhibitors which interfere with the response to GA3. The content of the inhibitors does not seem to be affected by the ageing of internodes or by the light treatment.

Pea plants have been very often used as objects for studying the role of hormones in growth, as well as the interaction of light and hormones in the growth process. Nevertheless, data concerning the content and metabolic changes of endogenous growth substances are still inadequate to permit the full understanding of their role in growth and morphogenesis. The remarkable effect of exogenous gibberellins on the growth of dwarf (Brian and Hemming 1955) and light-grown (Lockhart 1956) peas, led to the suggestion that these substances may be involved in the control of internode elongation. However, the effect of genetic or environmental factors on growth cannot be readily explained in terms of their effect on gibberellin content (Kende and Lang 1964; Jones and Lang 1968; Kende 1967; Köhler 1970, 1971). The assumption that endogenous inhibitors may also be involved in growth (Brian and Hemming 1958; Simpson and Wain 1961) seems to be supported by the recent discovery of abscisic acid (Isogai et al. 1967; Dörffling 1967; Barnes 1972), xanthoxin (Firn et al. 1972) and pisatin (Tamura et al. 1972) in pea stem tissue. We have studied the content of gibberrellin-like

* The results of this paper were presented at the International Conference on Natural Plant Growth Substances, 1972, Liblice, Czechoslovakia.

** Address: Takovska 43, 11000 Beograd, Yugoslavia.
substances and inhibitors in peas, as a function of the length of internodes, grown either in darkness, or irradiated briefly with red light. The present paper also reports some preliminary results concerning the possible identity of gibberellins and inhibitors found in the extracts.

**Material and Methods**

Pea plants (*Pisum sativum* L., cv. Alaska) were grown in darkness for seven d. Light treatment, when applied, consisted of five min red light (1.4 μW cm⁻² nm⁻¹ at 660 nm) given on the seventh d, at different time intervals before extraction.

At desired time, plants were quickly harvested and dipped into liquid nitrogen. Stems, or in most cases third internodes, were cut and extracted with cold methanol. After it had been shaken with light petroleum, methanol was evaporated almost to dryness, and the residue shaken with ethyl acetate at pH 2.8. For qualitative analysis of gibberellin-like substances, the ethyl acetate fraction of a large portion of material was chromatographed on a celite 545 phosphate-buffered column (Kende and Lang 1964) and eluted with dry ethyl acetate (ten 50 ml fractions), followed by acetone (two fractions of 200 ml each). The fractions containing gibberellins were collected and used for TLC.

For analyses of larger amounts of inhibitors, the ethyl acetate extract was separated into acidic and neutral fractions, by shaking with 2% sodium bicarbonate. The residue of the evaporated neutral fraction was applied onto a silica gel column (3.5 × 25 cm), which was eluted with benzene-ethyl acetate (2:1, v/v), according to Taylor and Burden (1970). Fractions of 20 ml were collected and those containing inhibitors were used for TLC.

However, for quantitative measurements of small samples of variously treated plants, stimulating and inhibiting substances had to be carefully separated. Besides, a large purification pro-

---

**Legend to Fig. 1**

- Ethyl acetate fraction, pH 2.8
- TLC System 1
- Fractions 1 to 3 (Acidic substances)
  - TLC System 3
    - Fractions 1 to 8 (Gibberellin-like substances)
      - Endosperm test
    - Fractions 9 to 10 (Acidic inhibitor)
      - Coleoptile test
      - Endosperm test + GA₃
      - Lettuce hypocotyl test
- Fractions 7 to 10 (Neutral inhibitors)
  - TLC System 2
    - Coleoptile test
    - Endosperm test + GA₃
    - Lettuce hypocotyl test

*Abbreviations used: ABA — abscisic acid, TLC — thin layer chromatography.*
GIBBERELLIN-LIKE SUBSTANCES AND INHIBITORS

Table 1
RF VALUES OF GIBBERELLIN-LIKE SUBSTANCES found in pea stem extracts

<table>
<thead>
<tr>
<th>Fractions from celite column</th>
<th>Adsorbent and solvent systems*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>2 to 4</td>
<td>0.0—0.1</td>
</tr>
<tr>
<td>5 to 7</td>
<td>0.0—0.3</td>
</tr>
<tr>
<td></td>
<td>0.4—0.6</td>
</tr>
<tr>
<td>10 to 12</td>
<td>0.0—0.3</td>
</tr>
</tbody>
</table>

Gibberellins

<table>
<thead>
<tr>
<th></th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA₁</td>
<td>0.10</td>
<td>0.48</td>
<td>0.54</td>
<td>0.06</td>
</tr>
<tr>
<td>GA₃</td>
<td>0.05</td>
<td>0.47</td>
<td>0.57</td>
<td>0.04</td>
</tr>
<tr>
<td>GA₄+₇</td>
<td>0.85</td>
<td>0.70</td>
<td>0.78</td>
<td>0.17</td>
</tr>
<tr>
<td>GA₅</td>
<td>0.83</td>
<td>0.70</td>
<td>0.88</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* (Stahl 1967).

3 — Kieselguhr G, 0-25 mm; equilibrated overnight in the lower phase of benzene-acetic acid-water (8 : 3 : 5, v/v/v), developed in the upper phase, 15 cm.

4 — Silica gel H, 0-25 mm, developed in chloroform-ethyl acetate — acetic acid (50 : 40 : 10, v/v/v), 10 cm.

5 — Silica gel H, 0-25 mm, developed in benzene-n-butanol-acetic acid (75 : 20 : 5, v/v/v), 10 cm.

6 — Silica gel H, 0-25 mm, developed in di-isopropylether-acetic acid (95 : 5, v/v), 10 cm.

procedure had to be avoided in order to minimize losses. Therefore, the ethyl acetate fraction obtained from 50 plant parts has been streaked directly to thin layers. A combination of preparative and analytical solvent systems was devised, permitting the virtual separation of gibberellin-like substances from both acidic and neutral inhibitors. These methods are outlined in Fig. 1.

Barley endosperm test was used for detection and measurements of gibberellin-like substances (Coombe et al. 1967). Inhibitors were detected by the same test in presence of GA₅, by oat coleoptile (Nitsch and Nitsch 1956) and lettuce hypocotyl tests (Frankland and Wareing 1960).

Results

Gibberellin-like Substances

The chromatography of gibberellin markers on the celite column has shown that GA₄+₇ appear in fraction one, GA₅ in fractions two and three, GA₁ in fraction nine and GA₃ in fraction eleven, the last two being not sharply separated. When the ethyl acetate fraction was chromatographed in the same system, three zones of activity were found, well separated from each other. One appears in fractions two and three, or three and four, the second in fractions five and six, or six and seven, and the third one in fractions ten to twelve. The three active fractions were collected and developed on thin layers with different solvents (Table 1). As can be seen, the first two fractions split in two, sometimes even three, active zones, indicating the presence of at least four or five different gibberellins in all of them.
Separate internodes have been extracted in several sets of experiments. It was found that the second internode contains considerable amounts of gibberellins only while the third one is still young, not exceeding 15 to 20 mm in length. When the third internode was longer, no more free gibberellins were found in the second one. Similarly, the total content of gibberellins in the third internode decreases as the internodes grow (Fig. 2).

When etiolated plants were exposed to red light for a short time, it was found that the level of gibberellins increased about 20 min after irradiation, but then rapidly fell down, so that two hours later the gibberellin-like activity was no more detectable (Fig. 3). The histograms show that the rise after 20 min and the loss of gibberellins after two h are common to all internodes. However, when the total content of gibberellins was calculated per mg of dry weight of tissue, it was found that in the internodes of 5, 15 and 25 mm, the gibberellin content was increased by 135%, 605% and 262% respectively, over the dark-grown controls. It should be mentioned that, when the third internodes are 5 mm in length, a certain increase in gibberellin content occurs in the second internodes too, so that the total content in the upper part of those stems is actually higher than that one calculated above.

Inhibitors

In some preliminary experiments we have tried to measure gibberellin-like substances in the whole ethyl acetate fraction and have found that the more

<table>
<thead>
<tr>
<th>Substance</th>
<th>Adsorbent and solvent systems*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Acidic inhibitor</td>
<td>0.90</td>
</tr>
<tr>
<td>Abscisic acid</td>
<td>0.90</td>
</tr>
</tbody>
</table>

* (Stahl 1967).
For solvent systems 3, 4 and 6 see legend to Table 1.
7 — Silica gel H, 0.25 mm, equilibrated overnight in the upper phase of carbon tetrachloride-acetic acid-water (8 : 3 : 5, v/v/v), developed in the lower phase + 10% ethyl acetate, 10 cm.
diluted the extract, the higher stimulation was obtained. This suggested the presence of substances usually referred to as “inhibitors interfering with the response to GAs” (KöHLR and LANG 1963). When acidic and neutral substances were separated, both fractions were found to contain inhibitors. The inhibitor from the acidic fraction closely corresponded to ABA in several TLC solvent systems (Table 2). In addition to its solubility properties and biological action, this may be taken as a strong suggestion of the identity of this substance with ABA.

When the neutral ethyl acetate fraction was chromatographed on a silica gel column, the inhibitory activity was found in fractions 37 to 47. These fractions were collected and applied to TLC. Biological tests revealed the presence of three substances, having the Rf values about 0.2, 0.5 and 0.8 respectively. All the three substances can be detected by their quenching

---

**Fig. 3.** Histograms of gibberellin-like activity in barley endosperm assay, of extracts from 50 third internodes. Length of internodes (horizontal rows): 5 mm (bottom), 15 mm (middle) and 25 mm (top). Light conditions (vertical rows): dark-grown plants (left), irradiated for 5 min with red light, extracted after 20 min (middle), irradiated as above, extracted after 120 min. TLC system 3 (see legend to Table 1).
ultra violet light and as orange spots, after spraying with 2,4-dinitrophenyl-hydrazine reagent (Fig. 4).

Biological properties of substances eluted from Rf 0.2 and 0.5 were compared with those of ABA, and found to be rather dissimilar. In the coleoptile test the dose-response curves for both inhibitors have a very steep slope, the inhibitors being actually toxic in the highest concentration (Fig. 5), while ABA, and presumably xanthoxin (Taylor and Burden 1972), do not produce a complete growth inhibition or toxic effects even in high concentrations.

The content of acidic and neutral inhibitors was also assayed in the extracts of separate internodes of different age, etiolated or after illumination. Neither of the biological tests used did point to any significant difference in the content of these substances. So it seems that no essential change in inhibitors' content occur during the elongation of the internodes, nor after irradiation.
Discussion

It is obvious that the methods applied for gibberellin analysis could not lead to their full identification. The only gibberellin conclusively proved in peas so far has been GA$_{20}$, found in pea pods (Komoda et al. 1968). There are also indications of the presence of GA$_1$ and GA$_5$ (Kende and Lang 1964; Jones and Lang 1968), although the later substance may be identical with GA$_{20}$ (Komoda et al. 1968). Other authors have also reported the occurrence of gibberellin-like substances chromatographically similar to GA$_1$/GA$_3$ or GA$_5$ (Köhler 1970; Šebánek 1966). In this work the chromatography on celite column showed the presence of gibberellins similar to GAs (fractions 2 and 3), but in TLC with four solvent systems the Rf values of fractions two and three and GA$_5$ were definitely different. Having no GA$_{20}$ as a marker, it was not possible to make the necessary comparison. Most of the gibberellins from the extract resemble GA$_1$/GA$_3$ in TLC, but at least some of them must be different according to their elution pattern from the column. The gibberellins found in celite fractions five to seven do not correspond to the markers used both in celite and in TLC, meaning that they are different from GA$_1$, GA$_3$, GA$_4$, GA$_5$, GA$_7$ and other gibberellins with similar Rf values. A similar fraction from celite column had been found in bean extracts (Crozier and Audus 1968), which were shown later on to contain probably GA$_1$ and GA$_{19}$, in addition to GA$_4$ (Crozier et al. 1971).

Obviously, further comparison cannot help in identifying unknown gibberellins. However, we may conclude that pea extracts contain four or five different gibberellin-like substances, which is more than expected in the beginning.

On the basis of quantitative measurements it seems that the internodes have the highest content in gibberellin-like substances at the onset of their elongation. The level of gibberellins may be further increased, 20 min after a short light treatment, more in young than in older internodes, which is followed by complete disappearance of gibberellins two hours later. It is difficult to discuss these data as we have little knowledge of what a certain amount of a substance actually means. Köhler (1971) found an inverse correlation of gibberellin content and growth in peas and suggested that the low content means their involvement in growth. It is possible that the high gibberelin content in young internodes enables them to elongate rapidly, and that the older tissues gradually lose gibberellins, which slows down their growth. The total disappearance of gibberellins two hours after irradiation cannot be explained at this moment. It is interesting to note that this period of gibberellin disappearance precedes the period of maximal growth inhibition which was found to occur in peas two to three hours after light treatment (Russel and Galston 1971).

The rise in gibberellins after a brief light treatment was reported before (Nešković and Konjević 1972) and is similar to a phenomenon observed also in cereal leaves (Reid et al. 1968; Loveys and Wareing 1971). The ability of younger internodes to accumulate more gibberellins is perhaps related to the specific phase of their growth at the time of irradiation. Thomson (1959) has shown that the response of pea internodes to the light treatment depends on their age, the very young internodes showing an
acceleration, the older ones only an inhibition of growth. It may be considered that this differential response is related to their different content of gibberellins after irradiation. However, experiments with short-term growth measurements would be needed to conclude whether the temporary rise in gibberellins has any influence on the subsequent growth rate.

The identity of the neutral inhibitors is unknown. In the series of recent papers, Taylor and Burden (1970, 1972) reported on the occurrence and structure of a neutral inhibitor, xanthoxin, a carotenoid derivative related to ABA. Applying the same methods, we have isolated the inhibiting zone on the silica gel column, but in TLC it was shown to contain three separate substances, only one of them having Rf similar to xanthoxin (0.5). However, in several points, the infra red spectra of the substances at Rf 0.2 and 0.5 are not in accordance with the proposed structure of xanthoxin or its derivatives. The mass spectrometry also suggests a higher molecular weight, probably 390. The possibility that these inhibitors are identical to pisatin, found in pea stem extracts by Tamura et al. (1972), was also considered. The comparison of infra red spectra of the inhibitors with the published data for pisatin (Perrin and Bottomley 1962) suggests again that such a possibility is highly improbable. The full chemical evidence, concerning inhibitors, which are still under study, will be published elsewhere.

Finally, our inhibitory substances have also different physiological properties than the neutral correlation inhibitor found in peas by Libbert and Liebenow (1964), since our substances are very active as inhibitors of coleoptile growth per se, i.e. in the absence of indolyl-3-acetic acid. The correlation inhibitor had under the same conditions very little activity.

The failure to find a correlation between the growth of internodes and the ABA-like substance is in agreement with the findings of some other authors (Kende and Kays 1971; Barnes 1972; Simpson and Saunders 1972). They also concluded that ABA plays no essential role in the regulation of stem growth in peas. Burden et al. (1972) showed that irradiation increases the content of xanthoxin in pea stem tissue. With different light treatment used, we were not able to confirm this for the neutral inhibitors. The fact that these inhibitors are rather toxic and yet found in young, actively growing tissue, suggests that they are probably separated in the cells from the growth controlling system, at least in the developmental phase of the plants under study.

Acknowledgement

The authors are very much obliged to Dr. D. Broadbent, ICI, England, for samples of GA_1, GA_4, and GA_5, as well as to F. Hoffman-La Roche, Switzerland, for a gift of abscisic acid used in these experiments.

References


GIBBERELLIN-LIKE SUBSTANCES AND INHIBITORS


TAMURA, S., IKEGAMI, S., KOMOTO, N., NOMA, M.: Occurrence of substances in dwarf peas inter-
MIRJANA NEŠKOVIĆ, T. SJAUŠ


Třetí internodia nebo celé stonky sedmidenních etiolovaných rostlin hraehu byly extrahovány a byl určován obsah látek typu gibberelinu a obsah inhibitorů. Extrakty obsahovaly 4 až 5 látek typu gibberelinu, některé z nich byly chromatograficky blízké GA3. V mladých internodiích byl zjištěn vysoký obsah gibberelinů a tento obsah se s prodlužováním internodií snížoval. Ozáření červeným světlem způsobovalo kvantitativní změny v obsahu gibberelinu, tyto změny též závisely na délce internodií. Extrakty obsahovaly též acidické a neutrální inhibitory, které interferují s reakcí na GA3. Obsah inhibitorů nebyl ovlivněn stářím internodií ani působením světla.