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Relationship between Light-induced Growth Inhibition and Acidic Growth Inhibitors in the Seedlings of Dwarf and Tall Cultivars of *Phaseolus vulgaris* L.

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Abstract : To clarify the role of endogenous inhibitors of growth in dwarf and tall bean plants, a search for specific inhibitors of growth was undertaken using the acidic substances of acetone extracts from the hypocotyls of dwarf (cv. Morocco) and tall (cv. Kentucky Wonder) bean seedlings (*Phaseolus vulgaris* L.). Two inhibitors were isolated from both cultivars and named A-1 and A-2, respectively, based on the order of their elution from a silica-gel column. Variations in the activity of the inhibitors in hypocotyls of both cultivars after the onset of red-light irradiation were determined using a bean bioassay, and the results were compared with those of red-light-induced inhibition of growth. The difference in the activity of A-1 and A-2 between the two cultivars was not great, and the changes with time in activity of A-1 and A-2 in hypocotyls of each cultivar did not reflect the light-induced inhibition of growth of hypocotyls of the same cultivar. These results suggest that acidic inhibitors, A-1 and A-2, are not involved in light-induced growth inhibition of the bean.
Key words : Growth inhibitor, Legume, Light inhibition, *Phaseolus vulgaris* L., Red light.

矮性および高性インゲンマメ芽生えにおける光生長抑制と酸性の生長抑制物質との関係 : 加藤　尚・塩田英二（香川大学農学部）

要　旨 : インゲンマメの矮性種（モロッコ）と高性種（ケンタッキーワンダー）の芽生えをアセトンで抽出して得た酸性画分について，生長抑制物質の検索を行うとともにその役割を調べた．その結果，両品種に共通する2種類の生長抑制物質がシリカゲルカラムにより分離でき，カラムの溶出順に A-1，A-2 と命名した．赤色光照射は矮性種芽生えの下胚軸の生長を著しく抑制する．この時の生長抑制物質の下胚軸での含量変動を，生物検定によって求めたところ，A-1 と A-2 の活性量は両品種間で大きな差はなく，これらの生長抑制物質の活性量の変動と芽生えの光生長抑制の度合との間には相関はなかった．これらの結果は，酸性の生長抑制物質，A-1 と A-2 はインゲンマメの光生長抑制に関与していないことを示唆している．
キーワード : インゲンマメ，生長抑制物質，赤色光，光抑制，マメ科植物．

Since the observation by Köhlor and Lang[8] that light-exposed dwarf peas contained high level of a growth inhibitor of stem extension, many investigators have reported that light-induced growth inhibition was caused by an increase in endogenous growth inhibitors[2,3,4,10].

In a recent study of the occurrence of neutral growth inhibitors in seedlings of dwarf and tall bean cultivars[5], three inhibitors were separated from both cultivars by silica-gel column chromatography that was eluted stepwise with benzene included increasing concentration of ethyl acetate. Among them an inhibitor eluted by 50～60% ethyl acetate in benzene caused considerable growth inhibition of hypocotyls of bean, and this activity was 2～3 times higher in the dwarf than the tall cultivar[5,6,7]. These findings suggested that this neutral growth inhibitor may play an important role in the growth inhibition of bean seedlings. However, a quantitative analysis of acidic growth inhibitors in these seedlings has not been performed as yet.

The purpose of the present experiments was to compare the levels of acidic inhibitors between dwarf and tall cultivars of *Phaseolus vulgaris* L., and to investigate whether the expression of photomorphogenesis can be explained in terms of the levels of these acidic inhibitors.

Materials and Methods

1. Plant materials

Two cultivars of bean (*Phaseolus vulgaris* L.) used were cv. Morocco (dwarf) and cv. Kentucky Wonder (tall). Seedlings were grown in plastic containers ($12\times25\times10$ (height) cm^3) filled with moist vermiculite in the dark at 25°C for 4 days including soaking period, as described previously[5]. The dark-grown 4-day-old seedlings (hypocotyl

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length, about 40 mm) were irradiated continuously with red light (2.3 Wm^{-2} at plant level) from above in illumination boxes at 25°C.

To measure the growth of the hypocotyls, the distance was determined between the base of the cotyledons and a small mark made with water-proof ink, which had been applied 20 mm below the base of the cotyledon at the beginning of the experiment. For quantification of growth inhibitors in hypocotyls, the same part of the segments was used as for measurement of growth of the hypocotyls. The segments were immediately frozen with dry ice and stored at −80°C until extraction of growth inhibitors. All handling after sowing was performed under a dim green safe-light (0.03 Wm^{-2}).

2. Extraction and separation of inhibitors

Bean hypocotyls were homogenized in ten volumes of cold acetone and the homogenate was filtered through filter paper (No. 1, Toyo). The residue was homogenized again in ten volumes of 80% (v/v) cold aqueous acetone, and filtered. The filtrates were evaporated *in vacuo* at 35°C to give an aqueous residue. The residue was redissolved in water and then adjusted to pH 7.5 with 1 M phosphate buffer and partitioned three times against an equal volume of ethyl acetate. The ethyl acetate phase was discarded. Subsequently, the aqueous phase was adjusted to pH 2.8 with 1 M phosphate and partitioned three times against an equal volume of ethyl acetate. The ethyl acetate phase was evaporated to dryness after drying over anhydrous Na_2SO_4 (acidic substances).

The acidic substances dissolved in acetone was adsorbed to celite (0.5 g, Celite 545; Nacalai), loaded on a column (2 cm i.d. × 30 cm) of silica-gel (40 g, Kieselgel 60, 70-230 mesh; Merck) and eluted stepwise with 100 mL per step of benzene that included increasing (10% per step, v/v) concentrations of ethyl acetate. After the eluting solvent had reached 100% ethyl acetate, the elution was completed with 200 mL methanol (100 mL $step^{-1}$).

3. Bean bioassay

Each fraction from the column was evaporated to dryness, dissolved in a small volume of acetone, added to a sheet of filter paper (No. 2, Toyo) in a 4.5-cm Petri dish and dried.

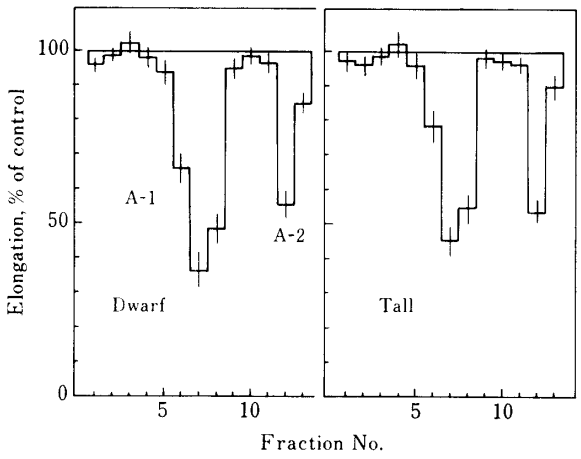


Fig. 1. Bean bioassay of fractions obtained after column chromatography of the acidic fractions isolated from hypocotyls of the two cultivars. All tested samples corresponded to 15 g fresh weight equivalent of hypocotyls. Values are means of results 10 segments ± SE.

Ten 10-mm segments which were excised below the hook of dark-grown 4-day-old seedlings of the dwarf cultivar were incubated in the Petri dish that contained 1.5 mL of a 0.05% (v/v) aqueous solution of Tween 20. After being kept in the dark at 25°C for 24 h, the lengths of the segments were then measured by use of a photographic enlarger, and the percentage elongation of growth was calculated by reference to the elongation of control segments.

4. Quantification of growth inhibitors

Plant material kept at −80°C was homogenized and the acidic substances was extracted. The acidic substances was chromatographed on a silica-gel column as described above. Fractions corresponding to each inhibitory activity (A-1, fractions 4-6 and A-2, fractions 12-13 in Fig. 1) were combined and tested in the bean bioassay at several dosages, and the percentage of any apparent inhibition was calculated with reference to the elongation of control segments. By applying each calculated value to the dose-response curve constructed for each inhibitor, as described previously[5], the activity of each inhibitor was expressed in "bean units". When more than two data points were available, the two values closest to 25% inhibition were used, and the mean of the two values was calculated.

All experiments were repeated three times and the results confirmed.

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Results and Discussion

1. Acidic growth inhibitors in bean hypocotyls

The acidic substances obtained from the hypocotyls of the dwarf cultivar was subjected to chromatography on silica-gel, and the fraction eluated each were tested in the bean bioassay (Fig. 1, left). Two peaks of inhibitory activity were detected in fractions 6-8 and 12-13, respectively, and the active principles were named A-1 and A-2 in order of their elution. Similarly, two peaks of activity were identified in the eluate of the acidic substances obtained from the hypocotyls of the tall cultivar (Fig. 2, right). These inhibitory fractions corresponded in terms of their elution profiles to A-1 and A-2 from the dwarf cultivar.

The column chromatographic property of A-2 corresponded to that of authentic ABA, and chromatogram of the A-2 fractions by HPLC (ODS AQ-325, 10×500 mm, YMC Co.; 15% acetonitrile (20 mM acetic acid), 2 mL min^{-1}; detected at 254 nm) contained *cis*-ABA (retention time 37.2 min) and a trace *trans*-ABA (41.7 min). These data suggest that the active principle of the A-2 fractions is ABA.

No promoter of growth was detected with this bioassay, but the results do not exclude the possibility that promoters are present in bean seedlings; the effects of promoters may have been masked by coexisting inhibitor(s) or, alternatively, such promoters may be ineffective in the assay.

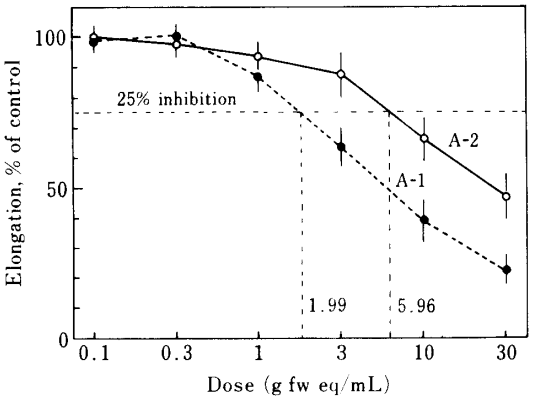


Fig. 2. Dose-response curves for A-1 and A-2 from standard samples in the bean bioassay. Values are means of results 10 segments ± SE.

2. Dose-response relationships in the bean bioassay

To estimate the inhibitory activity of A-1 and A-2, the active fractions obtained from hypocotyls of the dwarf cultivar were selected as standard samples and examined in the bean bioassay at several dosages (Fig. 2). The dose-response curves for these inhibitors were linear between 10 and 40% inhibition when the percentage inhibition was plotted against the logarithm of the dose.

When the activity that gives 25% inhibition in the assay is defined as one bean unit, the reciprocal of the dose that causes 25% inhibition can be taken as the specific activity, which is expressed in bean units per g fresh weight equivalent of the sample. For example, in figure 3, the dose on the abscissa means that 1 mL of medium contained a test sample derived from an indicated g fresh weight of hypocotyls; the dose of 1.99 g fresh weight equivalent of the standard sample of A-1 causes 25% inhibition in the assay, and the reciprocal of 1.99 gives the activity of A-1 as bean unit per g fresh weight equivalent. Therefore, 1 g fresh weight equivalent of the sample of A-1 contains 0.50 bean units. Thus, the specific activity of A-2 in the standard samples was calculated to be 0.17 bean units per g fresh weight equivalent.

3. Effects of red light

Figure 3 shows the time course of the

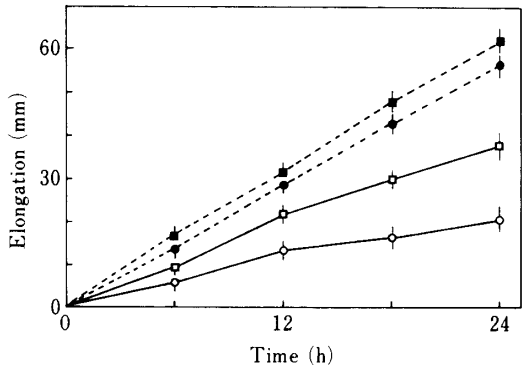


Fig. 3. Time courses of the growth of dark-grown seedlings of the two cultivars under continuous red light. Control plants remained in darkness. Means±SE of results from three replicates of 10 plants each are shown. Dwarf cultivar (○) in red light, (●) in darkness; tall cultivar (□) in red light, (■) in darkness.

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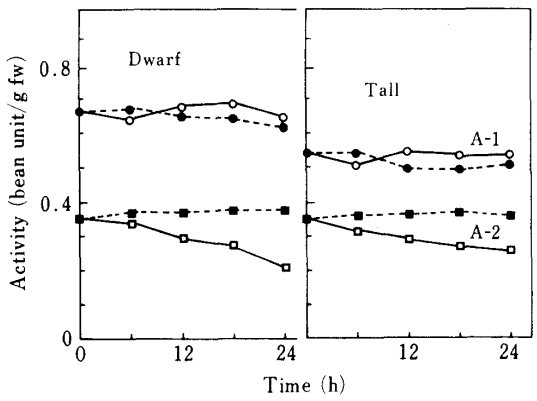


Fig. 4. Time courses of red-light-induced changes in the activity of A-1 and A-2 in segments of hypocotyls of dark-grown seedlings of the two cultivars. Each point represents a sample obtained from 50 plants. A-1, (○) in red light, (●) in darkness ; A-2, (□) in red light, (■) in darkness.

growth of hypocotyls of dark-grown seedlings of the two cultivars after the onset of continuous irradiation with red light. The growth of hypocotyls of both cultivars inhibited within 6 h after onset of red light, and the more noticeable change was shown in growth of the hypocotyls of the dwarf cultivar. Figure 4 shows the variations with time in the activities of the two inhibitors in segments of bean hypocotyls under these conditions. The difference in the activity of A-1 and A-2 between the two cultivars was not great. Furthermore, the activity of A-1 remained almost the same as the levels in the dark, and that of A-2 decreased gradually during the experiment.

The changes in activity of A-1 and A-2 observed in the hypocotyls of a given cultivar did not reflect the light-induced inhibition of growth of hypocotyls of the same cultivar. These results suggest that acidic inhibitors, A-1 and A-2, do not contribute to light-induced growth inhibition of bean seedlings, and agree with results previously reported supporting the conclusion that ABA and other acidic inhibitors are not involved in the light-induced growth inhibition of pea plants[1,9]. These findings together with the results in previous reports[5,6,7] indicate that the neutral growth inhibitor, not acidic inhibitor, may play an important role in growth inhibition of bean hypocotyls.

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