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Inhibitors from Carob (*Ceratonia siliqua* L.)

II. EFFECT ON GROWTH INDUCED BY INDOLEACETIC ACID OR GIBBERELLINS A_1, A_4, A_5, AND A_7 [1]

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ABSTRACT

Two inhibitory fractions (B_1 and C) from extracts of immature fruit of carob were tested for their ability to inhibit the action of indoleacetic acid (IAA) in three bioassays. There was no reduction of IAA-induced reactions in the *Avena* curvature test, abscission of debladed coleus petioles, or growth of cucumber hypocotyls. The highest ratio of inhibitor to IAA was 10,000 times greater than the ratio necessary to inhibit by 50% the growth caused by an equivalent amount of gibberellin A_3 in pea seedlings. At the highest concentration used, fraction C alone caused curvature of *Avena* coleoptiles. The inhibitory fractions appeared to enhance the effect of IAA in the cucumber test.

Concentrated whole extract and fractions B_1 and C were tested for reduction of growth caused by gibberellins A_1, A_4, A_5, A_7, and a neutral gibberellin-like substance from beans in the dwarf-5 maize bioassay. Each gibberellin was inhibited and required the same amount of inhibitor for a 50% reduction of the induced growth. The inhibiting effect could be completely overcome by increasing the amount of gibberellin while maintaining the same concentration of inhibitor. Fractions B_1 and C were also tested with gibberellins A_3 and A_4 in the cucumber hypocotyl test. Both inhibitory fractions reduced growth but were more effective against gibberellin A_3 than gibberellin A_4 in the assay. The ability to reduce gibberellin-induced growth and not reduce IAA-induced growth indicates that the inhibitors from carob have a greater specificity of action than that previously reported for any inhibitor.

Growth inhibitors are usually nonspecific in action and will block responses induced by any of several growth promotors. Coumarin, naringenin, and abscisic acid have been shown to inhibit responses due to indoleacetic acid, gibberellic acid (GA_3), and in some cases cytokinins (2, 7, 10, 17, 25). Extracts from carob will inhibit the growth induced by GA_3 in pea and maize seedlings (5, 6). The present paper reports on tests with IAA and with gibberellins other than GA_3.

MATERIALS AND METHODS

Carob Extract. Concentrated whole extract and the partially purified fractions B_1 and C were purified as described previously (4, 5). These fractions were separated from the other components by charcoal adsorption. Inhibitor C was then removed by differential extraction from aqueous solution into ether, and inhibitor B_1 was removed by subsequent extraction from the aqueous solution into ethyl acetate. Inhibitors B_1 and C are extractable to various degrees into diethyl ether from aqueous solutions at acid pH values, indicating that they may be weak acids (6).

Concentrations of inhibitory whole extract and fractions B_1 or C are given as the amount of extract obtained from a specified fresh weight of carob fruit. This figure is determined either for a total volume or for the volume given to each assay plant.

The amount of fraction B_1 or C used with a given amount of IAA is related to the activity of these inhibitors with gibberellic acid. In the dwarf pea bioassay, fraction B_1 or C extracted from 5 mg fresh weight of carob fruit will inhibit by 50% the growth induced by 0.05 μg of GA_3 (5). The ratio of extract to IAA is compared to this ratio in peas.

***Avena* Curvature.** The assay was used essentially as described by Went and Thimann (23). Curvature was allowed to develop for 90 min after application of the agar block. Phosphate buffer at a pH of 5.9 was used in preparing the blocks. IAA was used at a concentration of 50 or 100 μg/liter with or without the addition of fractions B_1 or C.

Coleus Petiole Abscission. The assay is similar to that used earlier (15, 24). Eight-week-old coleus plants, *Coleus blumei* Benth., which had been derived from cuttings were used. Each plant had from two to four branches, and the first full size leaf below the apex of each branch was used. Lanolin (0.2 ml) was added into half of a gelatin capsule. The leaf was debladed, and the half-capsule was introduced so that the petiole passed through the open end and the cut petiole surface was imbedded in the lanolin. Indoleacetic acid (0.1 μg per plant) or inhibitory fraction B_1 or C was added to the lanolin either separately or in combinations of IAA and inhibitor.

Cucumber Hypocotyl Elongation. The assay was adopted from Katsumi *et al.* (11). Seedlings of cucumber, *Cucumis sativus* L. cv. National Pickling, were used. About 100 seeds were soaked 2 to 3 hr in distilled water and then planted in a flat, containing a 1:1 mixture of soil and vermiculite. The flats were placed in a growth chamber which provided a 12-hr light period at 28 C and a 12-hr dark period at 22 C. The seedlings were used 6 days after planting when the hypocotyls were 25 to 30 mm long. Uniform seedlings were marked with India ink 20 mm below the cotyledonary node. The test solution (0.01 ml) in 95% alcohol was added to the apical bud of each seedling with a 0.01-ml pipette. The length of the marked hypocotyl unit was measured 3 days later.

Maize Assay. Seedlings of maize, *Zea mays* L., dwarf-5 mutant (d_5), were used as previously described (19, 20).

Gibberellins. Gibberellins A_1, A_4, A_5, and A_7 were supplied by P. W. Brian of Cambridge University. The "neutral gibberellin-like substance" was provided by L. Rappaport of the University of California, Davis. It was obtained from the Kentucky Wonder

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Table I. *Effect of Fractions B_1 and C on Avena Curvature*
Two concentrations of fraction B_1 and three concentrations of fraction C were each mixed with IAA in phosphate buffer, pH 5.9. Curvature was developed for 90 min.

IAA	Fraction B_1	Fraction C	Curvature[1]
μg/liter	*extract from g fresh wt/liter*		*deg*
0	0	0	0
50	0	0	14.0 ± 1.3
100	0	0	25.1 ± 1.7
50	1,000	0	15.7 ± 1.4
50	10,000	0	16.6 ± 1.2
0	0	0	0
50	0	0	14.5 ± 1.5
100	0	0	27.3 ± 2.2
50	0	1,000	12.4 ± 1.7
50	0	10,000	15.9 ± 1.0
50	0	100,000	26.1 ± 2.5
0	0	1,000	0
0	0	100,000	13.6 ± 1.1

[1] Average and standard error of 12 plants.

variety of bean seed by the method used to obtain a neutral fraction from potato tubers (8).

RESULTS

Effect of Inhibitors on *Avena* Curvature. Two concentrations of fraction B_1 and three concentrations of fraction C were each mixed with 50 μg of IAA and assayed in the *Avena* curvature test. Fraction B_1 did not reduce the curvature induced by IAA alone (Table I). At the highest concentration the ratio of B_1 to IAA was over 1000 times greater than the ratio needed to reduce by 50% the growth caused by an equivalent amount of GA_3 in pea seedlings. Fraction C also did not reduce the curvature induced by IAA (Table I). The combination of IAA and the highest concentration of fraction C resulted in a curvature that was greater than that induced by IAA alone. The highest concentration of fraction C alone gave a curvature equivalent to that caused by 50 μg of IAA. Whether this curvature was due to the inhibitory component or to an auxin contaminant has not been resolved. At the highest concentration the ratio of fraction C to IAA was over 10,000 times greater than the ratio needed to reduce by 50% the growth induced by an equivalent amount of GA_3 in pea seedlings.

Effect of Inhibitors on Coleus Petiole Abscission. Fractions B_1 and C were applied both separately and in combination with IAA to debladed coleus petioles. The addition of IAA alone retarded abscission by 2 days (Fig. 1). Fractions B_1 or C added with IAA had no effect on this retardation. Fractions B_1 or C alone had no effect on abscission. The ratio of inhibitor to IAA was 1000 times more than the ratio necessary to reduce by 50% the growth induced by an equivalent amount of GA_3 in peas.

Effect of Inhibitors on IAA-induced Growth of Cucumber. Four concentrations each of fractions B_1 and C were applied to cucumber seedlings. None of them affected hypocotyl growth. The same concentrations were mixed with IAA and assayed. Fraction B_1 enhanced the IAA response at all concentrations used (Fig. 2). Fraction C also showed indications of enhancing the IAA response. There was no evidence of inhibition of IAA-induced growth. At the highest concentration the ratio of fractions B_1 or C to IAA was 10 times greater than the amount needed to reduce by 50% the growth induced by GA_3 in the same system.

Effect of Inhibitors on Growth of Maize. Constant amounts of gibberellins A_1, A_4, A_5, and A_7 were mixed with decreasing amounts of whole extract and assayed. The extract clearly inhibited the growth induced by each of the gibberellins (Table II). With each gibberellin the extract from 5 mg (fresh weight) of carob fruit was able to reduce this growth by 50%. The neutral gibberellin-like substance was also used as a growth promoter in combination with the whole extract from carob. The growth induced by this gibberellin-like substance was also inhibited by the carob extract (Table II).

Similar experiments were performed in which gibberellins A_1, A_4, A_5, and A_7 (0.1 μg per plant) were mixed with decreasing amounts of fractions B_1 or C. These fractions also reduced the growth caused by each of the gibberellins. With both fractions B_1 and C, the extract from 50 mg (fresh weight) of carob fruit was the lowest amount able to reduce the gibberellin-induced growth by about 50%.

Reversibility of Inhibition. Increasing amounts of gibberellins were added to constant amounts of inhibitor in order to test the reversibility of the inhibitor effect. The whole extract was used

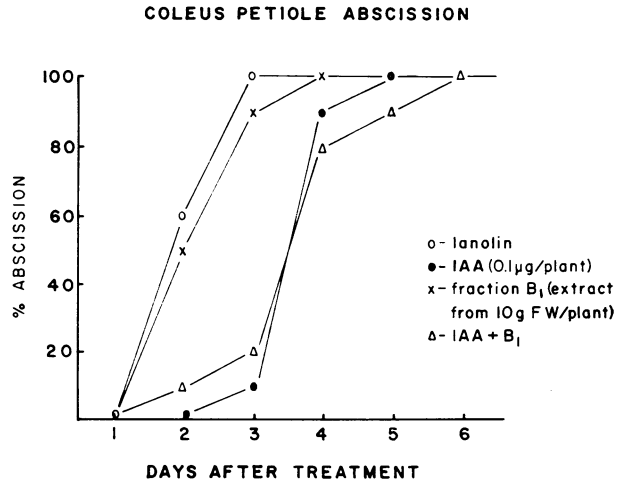

FIG. 1. Abscission of debladed coleus petioles. Half a gelatin capsule containing 0.2 ml of lanolin was applied to each petiole stump. The lanolin was used alone or with the addition of IAA, fraction B_1, or a combination of IAA and fraction B_1. Each point represents the average from 10 plants.

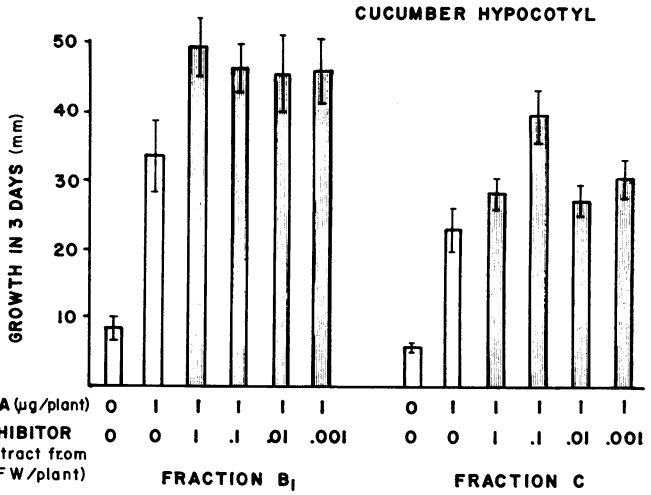

FIG. 2. Interaction of fractions B_1 or C with IAA in hypocotyl growth of cucumber. The growing tip of each seedling received 0.01 ml of ethanol alone or combined with IAA or with mixtures of IAA and different concentrations of fractions B_1 or C. Each point represents the average and standard error from 10 plants.

Table II. *Inhibition of Gibberellin-induced Growth by Whole Extract*

Seedlings treated with gibberellins or a combination of gibberellin and whole extract from carob.

Gibberellin		Whole Extract Concn	Leaf Sheath Length[1]	Inhibition
Kind	Concn			
	μg/plant	*extract from mg fresh wt/plant*	*mm*	*% reduction of gibberellin-induced growth*
A_1	0	0	23.1 ± 0.8	
	0.1	0	42.3 ± 2.2	
	0.1	50	27.5 ± 1.5	77
	0.1	5	29.9 ± 1.4	65
	0.1	0.5	40.9 ± 2.0	8
A_4	0	0	24.4 ± 0.9	
	0.1	0	55.7 ± 2.7	
	0.1	50	34.1 ± 1.5	69
	0.1	5	36.6 ± 1.8	61
	0.1	0.5	55.0 ± 2.4	2
A_5	0	0	19.6 ± 0.7	
	0.1	0	32.4 ± 1.2	
	0.1	50	20.7 ± 1.0	99
	0.1	5	23.8 ± 0.9	69
	0.1	0.5	31.1 ± 1.4	11
A_7	0	0	21.1 ± 0.9	
	0.1	0	54.3 ± 3.7	
	0.1	50	34.1 ± 1.2	61
	0.1	5	39.7 ± 3.9	45
	0.1	0.5	50.8 ± 2.6	11
Neutral gibberellin from Kentucky Wonder beans[2]	0	0	21.1 ± 0.5	
	10	0	29.3 ± 1.0	
	10	5	23.3 ± 0.8	73
	10	0.5	27.6 ± 1.3	21

[1] Average and standard error of 10 plants. Assays on dwarf-5 maize seedlings. Each seedling received a single application of 0.1 ml. Measurements were made 7 days after treatment.

[2] Concentration given as extract from g fresh wt/plant.

with gibberellin A_1 (Fig. 3). The amount of inhibitor was sufficient to reduce strongly the growth induced by the lower concentrations of gibberellin. The inhibition was completely reversed at the highest concentration of gibberellin. Similar results were obtained using whole extract with gibberellin A_5 and fraction B_1 with gibberellin A_4 or A_5. Other combinations were not tested.

Effect of Inhibitors on Gibberellin-induced Growth of Cucumber. Two gibberellins, A_3 and A_4, were used alone and with decreasing amounts of fraction C. In this assay GA_3 was about 1% as active as GA_4 (Fig. 4). Fraction C inhibited both gibberellins but appeared to be relatively more effective with GA_3 than with GA_4. A test in which gibberellins A_3 and A_4 were assayed with decreasing amounts of fraction B_1 gave similar results.

DISCUSSION

The auxin tests used here show a range in specificity. *Avena* curvature is sensitive only to translocatable auxins such as IAA while the cucumber hypocotyl test gives a similar response to both IAA and gibberellins. These assays were selected because in each case a whole seedling or plant was involved, thus making the assays more comparable to the shoot growth assays of peas

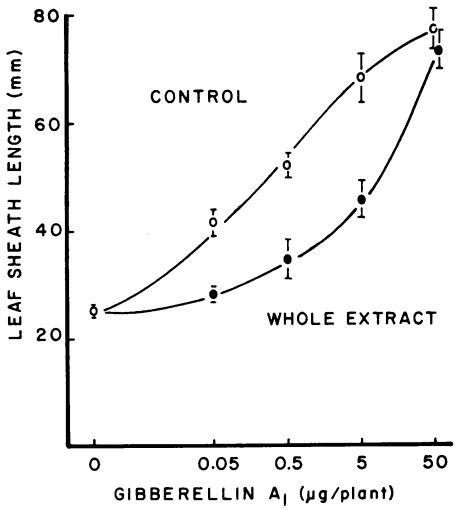

FIG. 3. The effect of gibberellin A_1 on the growth of maize seedlings in the presence and absence of whole extract. Each seedling treated with inhibitor received the extract from 5 mg fresh weight of carob fruit. Each point represents the average and standard error of 10 plants.

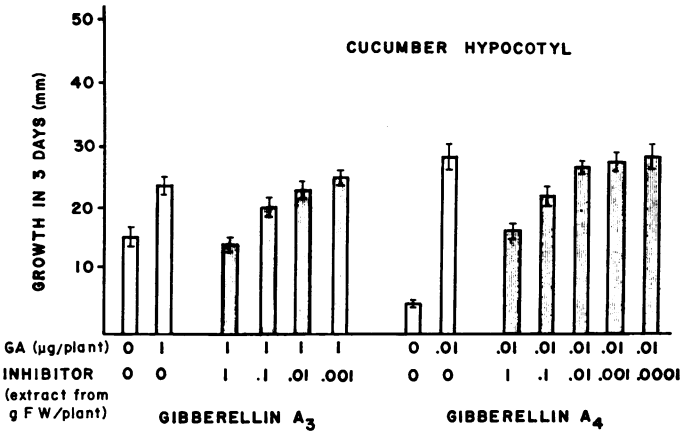

FIG. 4. Interaction of fraction C with GA_3 and GA_4 in hypocotyl growth of cucumber. The growing tip of each seedling received 0.01 ml of ethanol alone or combined with either gibberellin or a mixture of gibberellin and fraction C. Each point represents the average and standard error of 10 plants.

and maize, which have been the most frequently used for the carob inhibitors. The fact that the inhibitors are so very different in their effects against IAA and gibberellins in tests which have some similarity and which in the case of cucumber hypocotyl growth are identical indicates that the substances probably act quite differently with the two kinds of growth promotors.

Most plant growth inhibitors have been considered only in relation to auxin-induced phenomena (9). Those few which have been tested with other promotors have been found to be inhibitory. These are discussed by Leopold (12) and by Addicott and Lyon (1). The inhibitory system from carob extract appears to differ from other reported inhibitory substances in its specificity.

The enhancement of IAA-induced growth of cucumber by the carob inhibitors, especially fraction B_1, is reminiscent of the promotive effects of low concentrations of phenolic inhibitors with IAA (16, 22). The phenolic substances become inhibitory at higher concentrations. Specific activities cannot be compared between carob inhibitors and the phenolics because of the lack of information on the identity of the carob inhibitors.

The inhibitors from carob prevented growth induced by all four of the gibberellins tested and by the one neutral gibberellin-like substance. These results indicate that the inhibitors may have a broad spectrum of activity among the gibberellins. The inhibition of gibberellin-induced growth in maize is shown here to be overcome by adding more gibberellin. Similar results have also been reported with peas (5). Such results are consistent with the interpretation that the inhibitors are involved in a gibberellin mechanism.

There are marked differences in the activities of various gibberellins in bioassays. Gibberellins A_4, A_7, and A_9 are especially active in stimulating growth of cucurbits (3, 13). Gibberellin A_5 is only 15% as active as A_3 in the dwarf-1 maize assay (19). Just as different gibberellins cause different amounts of response, it might be expected that inhibitors of gibberellin-induced growth might vary in their activity with different gibberellins, and this seems to be the case here. Thus, it is interesting that the inhibitors are much more active against GA_3 than GA_4 in the cucumber hypocotyl assay.

In general, stem growth in intact plants is much more responsive to gibberellins than to auxins (21). Mature leaves and stems do not respond to gibberellins; however, they still respond to tropisms mediated by endogenous auxin. The same type of differential response has been shown in serial sections of the first leaf of wheat (25). The basal meristematic and next higher sections responded to GA_3 whereas the uppermost sections containing more mature tissues responded only to IAA, not to GA_3. Accumulation of an inhibitor which blocks gibberellin action but not auxin action might help explain these observations.

In a large number of species germination is stimulated by GA_3 whereas its stimulation by IAA is negative or questionable (14). The carob inhibitors have been extracted from immature seeds and fruit, and one of the fractions has been shown to suppress the appearance of α-amylase from the aleurone of barley (4). The location of the inhibitors and the reduction of an enzyme important in germination suggest that the inhibitors might normally be involved in regulating germination.

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