California State University, Northridge

THE INHIBITORY ACTION OF 5 TANNINS ON GROWTH INDUCED BY SEVERAL GIBBERELLINS

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology

by

Frederick Bardon Green

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The thesis of Frederick Bardon Green is approved:

Committee Chairman

California State University, Northridge

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ABSTRACT

THE INHIBITORY ACTION OF 5 TANNINS ON GROWTH INDUCED BY SEVERAL GIBBERELLINS

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The following tannins, Chinese gallotannin, 1, 2, 3, 4, 6-penta galloyl glucose, chebulinic acid, procyanidin dimer and procyanidin trimer were tested and found to inhibit seven different gibberellins in the dwarf pea assay. Endogenous growth was not affected. The gibberellins tested were GA₁, GA₃, GA₄, GA₇, GA₉, GA₁₃ and GA₁₄. The highest ratio of tannin to gibberellin tested (1000:1 by weight) inhibited from 65% to 95% of the induced growth for all tannins and all gibberellins tested. The procyanidin dimers and trimers are the first pure components of condensed tannins to be tested. They were the most potent inhibitors, causing up to 85% inhibition of GA₁₄-induced growth at a ratio of 10:1 (tannin-gibberellin). Inhibition could be completely reversed by increasing the amount of gibberellin. Complete reversal of the growth induced by 5 µg of gibberellin occurred with the addition of 5 µg of tannin per plant. These compounds thus qualify as native gibberellin antagonists.
INTRODUCTION

The presence of tannins has been well documented throughout the plant kingdom (Luft, 1928; Happick, 1954; Bate-Smith, 1962). Very early studies suggested that their structure and physiological action might be so complex as never to be determined (Michel-Durand, 1929). Since then, tannin chemistry has received appreciable attention and the structures of many tannins are now well known. Their function in the plant is much less well understood, although several suggestions have been advanced to explain their presence and role.

The earliest studies promoted the theory that tannins act to prevent disease and infection by plant parasites (Cook & Taubenhaus, 1912). This early research was subsequently supported by several studies with representatives of different plant families (Nienstaedt, 1953; Simura, 1941). Recent research with Rubus odoratus and Cornus canadensis has revealed that antitumor activity in these plants is induced exclusively by the tannins present (Fong, 1972). It has also been demonstrated that the presence of ellagitannins in the heartwood of Quercus alba may contribute to the inhibition of wood rotting fungus (Hart, 1972), but whether or not a correlation exists between specific disease resistance and the amount of tannin has been questioned (Zonsmeister, 1964; Swain, 1965). Based on personal research and a review of related experiments, Cruikshank (1964) found no specific correlation between disease resistance and the natural amount
of tannin present in plants. However, he did not preclude the possibility that tannin related compounds could be involved secondarily in disease resistance.

It has also been suggested that tannins might provide plants with the ability to resist extreme cold (Wood, 1929; Simura, 1941) since tea leaves show an increase in the amount of tannin present when subjected to cold conditions (Simura; 1941). This suggestion is also supported by the fact that plants containing high amounts of tannin are found more frequently in cold temperate regions than in the warmer tropics (McNair, 1930). However, other studies suggest increased amounts of tannin in plants subjected to cold shock may result from metabolic breakdown and not represent a defense mechanism of the plant (Wood, 1929).

Perhaps the most persistent research on tannins has been conducted in the area of plant growth regulation. Tannins have been shown to reduce the cambial activity in certain trees (Jacquiot, 1947). There have been some studies indicating that tannin inhibits germination and normal growth of seedlings (Freidrich, 1958; Forster, 1957), but still other studies have demonstrated that tannin promotes germination of seeds and seedling growth (Forster, 1957; Popoff, 1951). Gallic acid, one component of hydrolyzable tannins, has also been shown to inhibit the flowering activity in Kalanchoe blossfeldiana (Pryce, 1972). Other studies have suggested that tannins may be negative catalizers of oxidation and thus act as moderators of intracellular
oxidation (Lutz, 1928), and recent studies have shown that tannins act to reduce electron flow in photosystem II of plant chloroplasts (Schneider, 1971b).

Zinsmeister (1964) has shown that Chinese tannin and the tannin components, gallic acid and d-catechin, inhibit auxin-induced growth in much the same way. All reduce the amount of growth otherwise induced by indolacetic acid (IAA) in Avena coleoptile sections. Additional IAA will partially overcome the inhibition. Research conducted by Corcoran, et al. (1972) established specific, chemically defined tannins as GA₃ antagonists in that they will inhibit GA₃-induced growth, have no effect on endogenous growth, and the inhibition can be overcome with additional GA₃. The work was restricted to interaction with GA₃; no other gibberellins were used. The crystalline tannins used were all hydrolyzable tannins and only crude mixtures of condensed tannins were employed. The inhibitory action of three hydrolyzable tannins was tested and shown to be reversed with additional amounts of GA₃.

The purpose of this study is to explore the interaction of some previously tested tannins with several gibberellins other

*Nomenclature for gibberellins is that recommended by Lang (1970), "The abbreviation GA(s) stands for gibberellin(s) in general. Individual gibberellins are designated as GA₁, GA₂, etc. GA-like substances are substances occurring naturally and causing responses typical of known GA's, but not yet known chemically."
than GA₃. Previously untested tannins, including two chemically defined components of condensed tannins were tested with several gibberellins. Evidence to classify the inhibition as antagonistic was obtained by testing the reversibility of growth suppression with additional gibberellin.
MATERIALS AND METHODS

The dwarf pea assay used to measure gibberellin activity was conducted as originally described by Corcoran and West (1968) and later modified (Corcoran, 1970a). Seeds of peas, Pisum sativum, L., cultivar Little Marvel, were planted in greenhouse flats containing a 1:1 mixture of vermiculite and soil, and placed in incubators at 20°C with an alternating day and night cycle of twelve hours. The seedlings were treated 5 to 7 days after planting, at which time the shoot length was 15mm to 20mm and the leaves still encircle the growing tip. The test solution was applied to the apical region of the plant with a .01ml pipet. Measurements of shoot length were obtained 7 days after treatment by measuring the length from the lowest stem bract to the epicotyl. From these lengths all pertinent calculations involving growth and inhibition were made.

Tannins

Tannins were supplied by Dr. E. Haslam, University of Sheffield and included Chinese gallotannin, \( \beta - 1, 2, 3, 4, 6 \)-penta galloyl glucose, and procyanidin dimers and trimers (see figures 1-4 for structures). Chebulinic acid was also supplied and was recrystallized from water prior to use.

The tannins were weighed and dissolved in either 1 ml or 4 ml water. In order to facilitate solution, the mixtures were heated and one drop of .05% Tween 20 (Sorbitan polyoxethlene monolaurate) was added. A series of 10 fold dilutions were made
Figure 1

Structure of Chinese gellotannin which is the principal constituent of Chinese galls (*Quercus infectoria*)
The structure of \( \Phi-1, 2, 3, 4, 6 \)-penta galloyl glucose, a hydrolyzable gallotannin.
Figure 3

The structure of chebulinic acid, a hydrolyzable ellagitannin.
Figure 4

The structure of condensed tannin components, procyanidin dimers, two possible isomers (1a, 1b); procyanidin trimer, one possible isomer (2).
with water from the original concentration. Each of these solutions was finally diluted with an equal amount, by volume, of gibberellin or water so that the concentration applied per plant was from the series .5ug/.01ml, 5ug/.01ml and 50ug/.01ml.

**Gibberellins**

Gibberellins A₁, A₄, A₇, A₉, A₁₃ and A₁₄ were received from Dr. J. Mac Millan, University of Bristol. Their structures can be seen in figure 5. The gibberellins were weighed and dissolved in Tween 20 (.05%) to give a concentration of 100ug/.01ml. A series of 10 fold dilutions was made with the Tween 20 solution to give a concentration range. Each of these mixtures was diluted with an equal volume of the tannin solution or water so that the concentration of gibberellin applied per plant was 50ug/.01ml, 5ug/.01ml or .05ug/.01ml.

The final experiments with GA₄ which attempted to reverse inhibition with additional gibberellin were performed with another sample of GA₄ also sent by Dr. J. Mac Millan. It consisted of a mixture of 85% GA₄ and 15% GA₇.

The GA₃ was purchased as the sodium salt from Calbiochem, Los Angeles. It contained 20% inert ingredients, and weights were calculated to obtain the same concentration of GA₃ as in the pure samples of the other gibberellins.
Figure 5

Chemical structure of gibberellins used for this study.
RESULTS

Tannins and Gibberellin-Induced Growth

Five tannins were tested for their ability to inhibit growth induced by any of 7 gibberellins in the dwarf pea assay. Gibberellin was dissolved in Tween 20 solution and applied separately or combined with tannin. The ratio by weight of the tannins to gibberellins was 1:1, 10:1, 100:1 and 1000:1. All tannins tested reduced growth caused by the gibberellins. The amount of inhibition was generally similar to that reported previously against GA3-induced growth.

The procyanidin dimers and trimer exhibited the highest amount of inhibition found (figs. 6-7). A ratio between 1:1 and 10:1 (trimer-gibberellin) was sufficient to cause a 50% reduction of growth of GA4 and GA14. The dimer produced the same reduction of growth induced by GA1 and GA3 at a ratio between 100:1 and 1000:1.

Chinese gallotannin and 1, 2, 3, 4, 6-penta galloyl glucose exhibited almost identical inhibition with all gibberellins tested. They reduced 50% of the growth induced by GA2, GA9 and GA14 when used at a ratio of about 10:1. GA3 and GA7 require a level of 100:1 to cause 50% inhibition. The highest ratio of tannin, nearly 1000:1, was required to cause a 50% inhibition of GA1-induced growth (figs. 8-9).

Chebulinic acid exhibited perhaps the most varied effects on gibberellin. It acted to inhibit 50% of the induced growth of GA3.
Figure 6

Effect of procyanidin dimers on growth induced by several GA's. Seedlings received 0.05 or 0.5 \( \mu \text{g} \) GA and three concentrations of tannin. Inhibition is given as percentage reduction of the gibberellin-induced growth. The data represent the average values from two replica runs. Each run consisted of the three inhibitor concentrations mixed with gibberellin and each mixture was assayed on 10 seedlings.
Figure 7

Effect of procyanidin trimers on growth induced by several GA's. Seedlings received 0.05 or 0.5 µg GA and three concentrations of tannin. Inhibition is given as percentage reduction of the gibberellin-induced growth. The data represent the average values from two replica runs. Each run consisted of the three inhibitor concentrations mixed with gibberellin and each mixture was assayed on 10 seedlings.
Figure 8

Effect of chinese gallotannin on growth induced by several GA's. Seedlings received 0.05 or 0.5 μg GA and three concentrations of tannin. Inhibition is given as percentage reduction of the gibberellin-induced growth. The data represent the average values from two replica runs. Each run consisted of the three inhibitor concentrations mixed with gibberellin and each mixture was assayed on 10 seedlings.
Figure 9

Effect of β-1, 2, 3, 4, 6-penta galloyl glucose on growth induced by several GA's. Seedlings received 0.05 or 0.5 µg GA and three concentrations of tannin. Inhibition is given as percentage reduction of the gibberellin-induced growth. The data represent the average values from two replica runs. Each run consists of the three inhibitor concentrations mixed with gibberellin and each mixture was assayed on 10 seedlings.
Figure 10

Effect of chebulinic acid on growth induced by several GA's. Seedlings received 0.005, 0.05 or 0.5 µg GA and three concentrations of tannin. Inhibition is given as percentage reduction of the gibberellin-induced growth. The data represent the average values from two replica runs. Each run consisted of the three inhibitor concentrations mixed with gibberellin and each mixture was assayed on 10 seedlings.
at a concentration of 100:1. The growth of $\text{GA}_{14}$ was reduced by the same amount at the low level of 10:1. The result with $\text{GA}_{1}$ was perhaps the most unusual. Using 0.05 µg $\text{GA}_{1}$ per plant a ratio of 1000:1 (chebulinic acid-$\text{GA}_{1}$) inhibited growth by only 25%.

Growth induced by $\text{GA}_{3}$ and $\text{GA}_{14}$ is more easily inhibited by the tannins tested than growth induced by other gibberellins. A ratio of 10:1 causes inhibition of 45% or more in 7 out of 10 combinations. The dimers and trimers are particularly inhibitory with reductions of up to 86% at a ratio of 10:1.

**Reversibility of Inhibition**

If the inhibitor and the promoter are acting on the same physiological system, the growth reduction should be reversible with additional gibberellins. Increasing amounts of gibberellin were added to a constant amount of tannin to test reversibility.

The gibberellins used were $\text{GA}_{1}$, $\text{GA}_{4}$ and $\text{GA}_{14}$ in amounts ranging from 0.005 µg per plant to 5 µg per plant. $\text{GA}_{3}$ was also used in amounts ranging from 0.005 µg per plant to 50 µg per plant. The tannins tested were procyanidin dimer and trimer, chebulinic acid and 1, 2, 3, 4, 6-penta galloyl glucose. These tannins were applied uniformly at a concentration of 5 µg per plant. Data for the combinations of tannin and gibberellin can be seen in figures 11-17. The inhibition was completely reversed by the highest concentrations used of $\text{GA}_{1}$, $\text{GA}_{3}$ and $\text{GA}_{4}$. At a concentration of 5 µg per plant these tannins completely reversed the inhibition. The growth reductions caused by procyanidin dimer was not reversed by 5 µg of $\text{GA}_{14}$. This result is not necessarily at variance with
Figure 11

Effect of gibberellin A₁ on the growth of pea seedlings in the presence or absence of 5 μg procyanidin dimer per plant. Each seedling received a total volume of .01 ml of test solution. Each point represents the mean and standard error of 10 seedlings.
Effect of gibberellin A₁ on the growth of pea seedlings in the presence or absence of 5μg chebulinic acid per plant. Each seedling received a total volume of .01ml test solution. Each point represents the mean and standard error of 10 seedlings.
Chebulinic acid
Figure 13

Effect of gibberellin A₃ on the growth of pea seedlings in the presence or absence of 5 µg procyanidin trimer per plant. Each seedling received a total volume of .01ml of test solution. Each point represents the mean and standard error of 10 seedlings.
Gibberellin A₃ (μg/plant)

Control

Procyandin trimer

Shoot Length (mm)
Figure 14

Effect of gibberellin A₄ on the growth of pea seedlings in the presence or absence of 5 μg of β-1, 2, 3, 4, 6-penta galloyl glucose per plant. Each seedling received a total volume of .01ml of test solution. Each point represents the mean and standard error of 10 seedlings.
Gibberellin A$_4$ (μg/plant)

Shoot Length (mm)

- Control
- 1,2,3,4,6-penta galloyl glucose
Figure 15

Effect of gibberellin $A_4$ on the growth of pea seedlings in the presence or absence of 5μg chebulinic acid per plant. Each seedling received a total volume of 0.01ml of test solution per plant. Each point represents the mean and standard error of 10 seedlings.
Gibberellin A₄ (ug/plant)
Figure 16

Effect of gibberellin $A_{14}$ on the growth of pea seedlings in the presence or absence of 5μg procyanidin trimer per plant. Each seedling received a total volume of .01ml of test solution. Each point represents the mean and standard error of 10 seedlings.
Gibberellin $A_{14}$ (ug/plant) vs. Shoot Length (mm). The graph compares the control treatment with the Procyanidin trimer treatment. The x-axis represents the concentration of Gibberellin $A_{14}$ in micrograms per plant, ranging from 0 to 5. The y-axis represents the shoot length in millimeters, ranging from 0 to 220. The data points show a marked difference in shoot length between the control and Procyanidin trimer treatments, with the latter showing a more pronounced growth effect at higher concentrations.
Figure 17

Effect of gibberellin $A_{14}$ on the growth of pea seedlings in the presence or absence of 5μg procyanidin dimer per plant.
Each seedling received a total volume of 0.01ml of test solution.
Each point represents the mean and standard error of 10 seedlings.
the other results for two reasons. First, previous reversal studies with GA₃ and tannins (Corcoran et al., 1972), and GA₃ with carob extracts (Corcoran & West, 1968) usually required 50µg GA per plant to reverse a similar amount of inhibitory activity. Second, it has already been demonstrated that GA₁₄ is more susceptible to inhibition than most other gibberellins tested, so it might be expected that larger amounts of this gibberellin would be required to overcome inhibition.
DISCUSSION

It was previously shown that several tannins are GA₃ antagonists because they can successfully inhibit the action of GA₃, have no effect on endogenous growth and their effect is reversible with additional GA₃ (Corcoran, et al., 1972). A degree of specificity is suggested by the fact that on cucumber seedling tests, tannins will reduce the growth caused by GA₃ but not that caused by indole acetic acid. Evidence presented here confirms the prior work with GA₃ and demonstrates, in addition, that tannins are antagonists of a variety of chemically different gibberellins. While the role of tannins in plants is unclear, it has recently been suggested that they may play a growth-regulatory role by inhibiting growth caused by gibberellin in the plant (Corcoran, et al., 1972). Three tannins not previously tested in this system, including two pure components of condensed tannins, were also found to be inhibitory to the growth induced by several gibberellins. Crystalline tannin representing three of the four major groups of tannins have now been tested and found to be gibberellin antagonists. The antagonistic action of several tannins against several gibberellins demonstrates the generality of this phenomenon and lends support to the idea that tannins may be involved in the normal control of plant growth.

In two cases a tannin was tested at two concentrations of the same gibberellin and the results were different in each case. Chebulinic acid was mixed with GA₁ at concentrations of .05μg per plant and .005μg per plant. At tannin to gibberellin ratios of
ratio to 1,000:1 did not give a greater reduction. 1, 2, 3, 4, 6-penta galloyl glucose was also used with two concentrations of \( \text{GA}_{14} \), 0.05\( \mu \text{g} \) per plant and 0.5\( \mu \text{g} \) per plant. In this case the same ratios of inhibitor to promotor gave very similar results.

The difference in results with the two concentrations of \( \text{GA}_{14} \) suggests the tannin is acting to block a specific amount of growth, while the similarity of results with two concentrations of \( \text{GA}_{14} \) suggests the tannin may be acting directly against the gibberellin molecule. Whether either of these possibilities is correct cannot be determined at this time.

While the mechanism of action of tannins as growth inhibitors is unknown, there is some evidence suggesting a direct involvement with gibberellin. An antagonist, particularly in view of the reversibility of its effect, would be expected to be closely involved with gibberellin function. In analogy with enzyme studies, tannins give experimental results similar to those of competitive inhibitors. Tannins could not be competitive inhibitors of gibberellins because the chemical structures of the two groups of substances are so dissimilar. However, as Lockhart (1962) has pointed out, in a whole plant growth system it is usually experimentally impossible to differentiate between inhibitors that compete with the promotor for an active site, and inhibitors which directly react with, and thus tie up, the promotor or its precursor. Although there is no direct experimental confirmation, reactions involving gibberellin or a precursor would be consistent with the results thus far available.
Tannins are generally classified into two main groups, hydrolyzable and condensed. The hydrolyzable tannins are further divided into gallotannins and ellagitannins on the basis of the products released upon hydrolysis. When hydrolyzed, gallotannins commonly yield gallic acid and glucose, whereas ellagitannins also yield ellagic acid. Of the tannins used in this study, Chinese gallotannin and 1, 2, 3, 4, 6-penta galloyl glucose are gallotannins. Chinese gallotannin is the principal constituent of Chinese gall (Quercus infectoria) and is a heptagalloyl derivative. The only ellagitannin represented in this study was chebulinic acid.

The condensed tannins differ from the hydrolyzable tannins in that they contain only a phenolic nucleus. These tannins are formed by polymerization of flavans to form complex structures and are often referred to as flavolans (Swain, 1965). There are two main divisions, those formed from flavan-3-ols, such as catechin, and those formed from flavan-3, 4, diols such as leucocyanidin. Specifically, the procyanidin dimers and trimers used are flavan-3-ol derivatives, and have also been termed proanthocyanidins (Haslam, 1972). Possible isomers of these tannins can be seen in figure 4. It should be noted that the procyanidin trimer may show several configurations since the epicatechin unit of the trimer may link to the 6 or 8 position of the flavan-3-ol unit (Haslam, 1972).
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