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Endogenous Gibberellins of a Radiation Induced Single Gene Dwarf Mutant of Bean[1]

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Abstract. **The distribution of endogenous gibberellins in Dwarf-1, a single gene dwarf mutant of Mexico 80-R red beans, was studied. Parallel extraction and fractionation of seeds of this mutant and those of a normal homozygous line followed by thin layer chromatography and bioassays using *Rumex obtusifolius*, wheat seed endosperm and dwarf bean plants revealed that a stem elongation control factor was contained in the non-acidic fraction from normal, but not from Dwarf-1, seeds. It was concluded that the single gene mutation causes a block either in gibberellin precursor formation or in production of a non-acidic fraction gibberellin-like substance.**

Moh and Alan (9) have isolated a line of dwarf mutants from gamma irradiated red beans and have shown by segregation studies that the phenotypic expression is recessive to the normal with single gene control. Subsequent work by these authors (10) revealed that the dwarfism phenomenon may be reversed by the application of an aqueous spray of gibberellic acid to the leaves, implying that a lack of hormone may account for the dwarfing. The gibberellic acid treatment induces internodal elongation and leaf expansion, and dwarf mutant plants subjected to optimal levels of the hormone are indistinguishable from plants of the normal line.

Plants of this particular line of mutants (designated Dwarf-1) are characterized by formation of successively more compacted internodes above an elongated hypocotyl. Because of this, vertical growth ultimately ceases. A similar situation has been observed in dwarf corn, except that the terminal node does elongate (6). The leaves of the Dwarf-1 plants are dark green, thickened, and not fully expanded (13). A comparative view of the normal and dwarf plants is shown in figure 1.

The present study was initiated on the premise that there was a gibberellin imbalance in the mutant line. A parallel experimental series was run, using the Dwarf-1 versus a normal homozygous parent line.

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[2] The data presented herein are taken from a thesis presented to the Graduate School of the IIAS by the senior author in partial fulfillment of the requirements for the degree of Master of Science.

Materials and Methods

Preparation of Extracts. The dwarf bean used in this study is a mutant of the red bean, *Phaseolus vulgaris* L., var. Mexico 80-R, which was developed by Moh and Alan (9). Plants of this mutant line, when subjected to an aqueous leaf spray of 32 mg/l gibberellic acid, undergo stem elongation and leaf expansion, reverting to the normal pheno-type. Seeds to be extracted were obtained from greenhouse grown plants treated with gibberellic acid to provide sufficient yields. No carry-over of the growth substance was evident when plants from seed of treated *vs.* untreated plants were measured for stem elongation and leaf expansion after having been grown together under identical conditions. Extraction of gibberellins was made from both mature seeds and green plant material.

The seeds were extracted according to the methods outlined by Hashimoto and Rappaport (4). They were ground in a Waring Blendor in 50% methanol, using 300 ml for each 100 g of air-dried seed, filtered, and the alcohol portion of the filtrate was evaporated off under vacuum. The remaining aqueous phase was adjusted to pH 7.5 with $NaHCO_3$ and extracted several times with ethyl acetate. These combined ethyl acetate fractions were labeled the non-acidic fraction. The water phase was then acidified with H_3PO_4 to pH 3.0 and re-extracted with ethyl acetate to yield the acidic ethyl acetate fraction. Finally, the water phase was again extracted with *n*-butanol to give the acidic butanol fraction. Any water remaining in the organic solvents was removed by passing the fractions through a column of anhydrous Na_2SO_4.

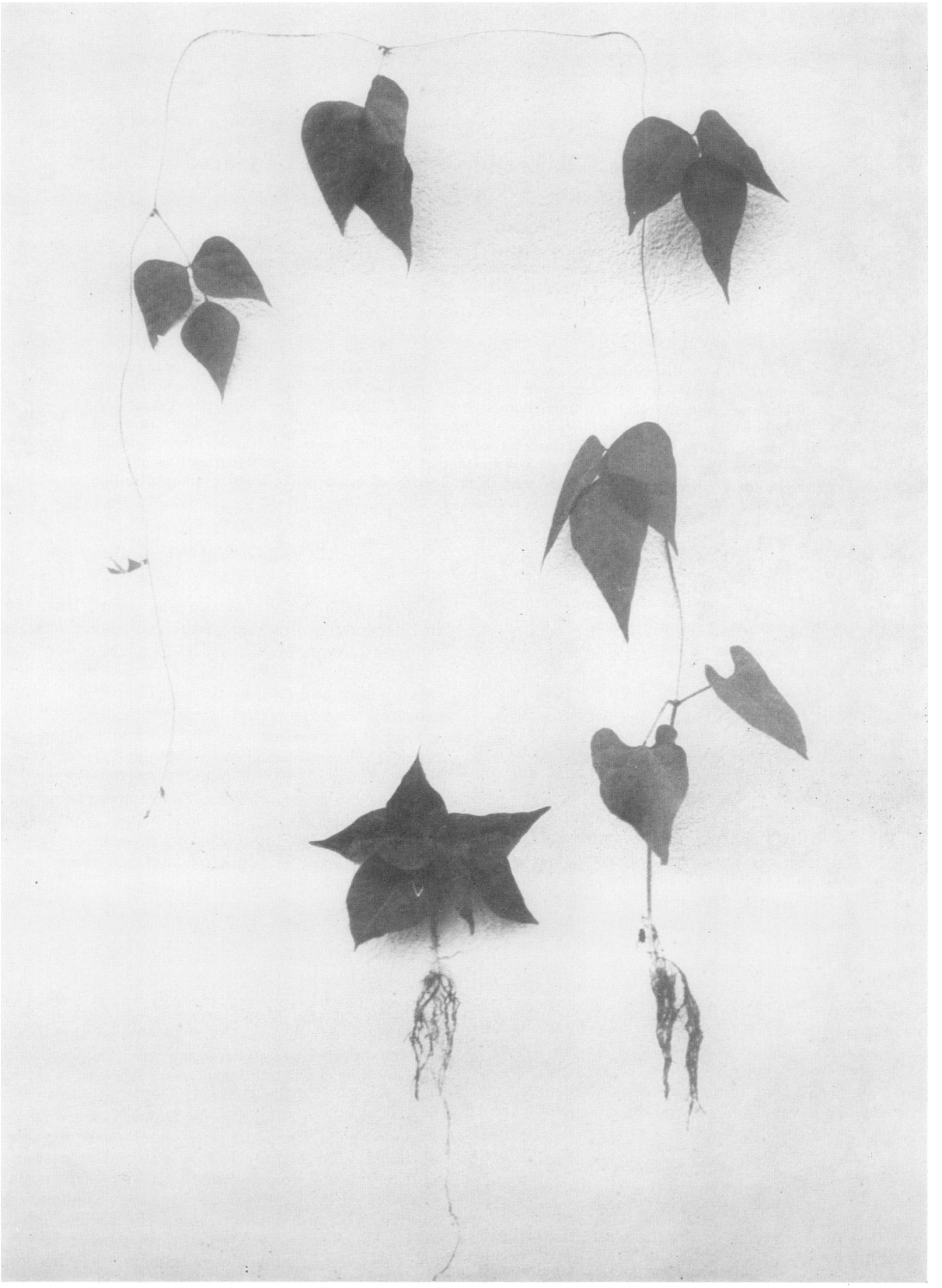

FIG. 1. A comparison of 28 day-old plants of the normal and Dwarf-1 mutant.

For the green plant material extractions, above ground portions of 21 day-old bean plants were killed by freezing in liquid nitrogen and extracted directly in ethyl acetate, using McComb and Carr's methods (7.) No further fractionation of these extracts was carried out.

All organic extracts obtained were reduced under vacuum to near dryness and either taken up in 10 ml of distilled, deionized water prior to use in the bioassays or used directly for the chromatographic separations.

Biological Tests. Three separate bioassays were employed in this study, the *Rumex* leaf disc test, the wheat endosperm test, and the dwarf bean mutant test. The first of these is based on the prevention of loss of chlorophyll by low concentrations of gibberellin in excised mature leaf discs of *Rumex obtusifolius* L. Plant material was collected from the slopes of Cerro de la Muerte mountain in Costa Rica and was propagated in pots in the greenhouse by root cuttings. The procedures of Whyte and Luckwill were in general followed (15), with the addition that it was found necessary to cut the discs under water to realize a meaningful response to the hormone.

The second bioassay was based on that of Nicholls and Paleg (12) in which gibberellin-induced reducing sugar production from starch in the endosperm of grain seeds is measured. Wheat seeds of the variety Tiba obtained from Ecuador instead of the usual barley were used because of a lack of an adequate source of the latter. Reducing sugars were measured by the methods of Nelson (11) and Somogyi (14).

The third test was the application of the extracts to Dwarf-1 plants. Greenhouse grown seedlings 21 days old were treated with the diluted extracts containing 0.05 % v/v Tween 20 applied as a foliar spray at the rate of 25 ml per plant. The total increase in height after 30 days as compared with non-treated controls served as the criterion of response to the gibberellin-like substances.

Chromatographic Separations. The extracts were spotted onto Silica Gel G thin layer plates and developed in benzene:*n*-butanol:acetic acid (70:25:5) after the procedures of McMillan and Suter (8). The presumed gibberellins were visualized by first examining the plates under ultraviolet light. They then were sprayed with sulfuric acid:water (10:30), heated and re-examined under the ultraviolet light to observe the appearance of fluorescent spots.

Results and Discussion

Before commencing with the assay of the various extracts obtained from the bean material, a series was run with known concentrations of gibberellic acid (10^{-5} to 1 mg/l) on each of the 3 bioassays. Regression analysis (fig 2) revealed that the *Rumex* test gave more reproducible results than did the wheat endosperm assay, probably because of variation in the wheat seeds (2). The dwarf bean test was shown to lack sensitivity below 10^{-2} mg/l.

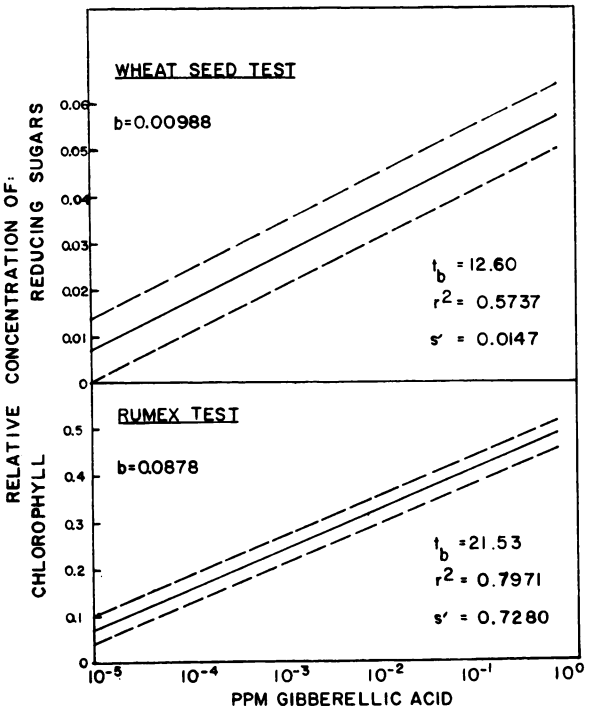

FIG. 2. Regressions of relative concentrations of chlorophyll or reducing sugars on gibberellic acid levels in the *Rumex* leaf disc and wheat endosperm bioassays. The 95 % confidence range is delineated by the broken lines.

Similar results for both the *Rumex* and wheat endosperm bioassays were obtained when the acidic ethyl acetate, acidic butanol and non-acidic fractions from the mature seed extracts were tested (fig 3, B and C). The greatest activity in both normal and dwarf bean extracts appeared in the acidic butanol fraction, followed by the acidic ethyl acetate and non-acidic fractions. In general, the response to the extracts of the dwarf seeds was qualitatively similar but about 25 % less than that of the normal line.

It might be concluded from these data that the dwarfing was a result of merely lesser amounts of growth hormones. However, examination of the data from the dwarf bean assay (fig 3A), revealed that substances present in the non-acidic fraction of the normal line were the most efficacious in causing stem elongation in dwarf plants. Neither of the acidic fractions of the normal seeds, nor any of the extracts of the dwarf seeds had a significant effect on stem elongation in the dwarf plants.

The differences in response of the *Rumex* and wheat bioassays to ethyl acetate extracts of stems

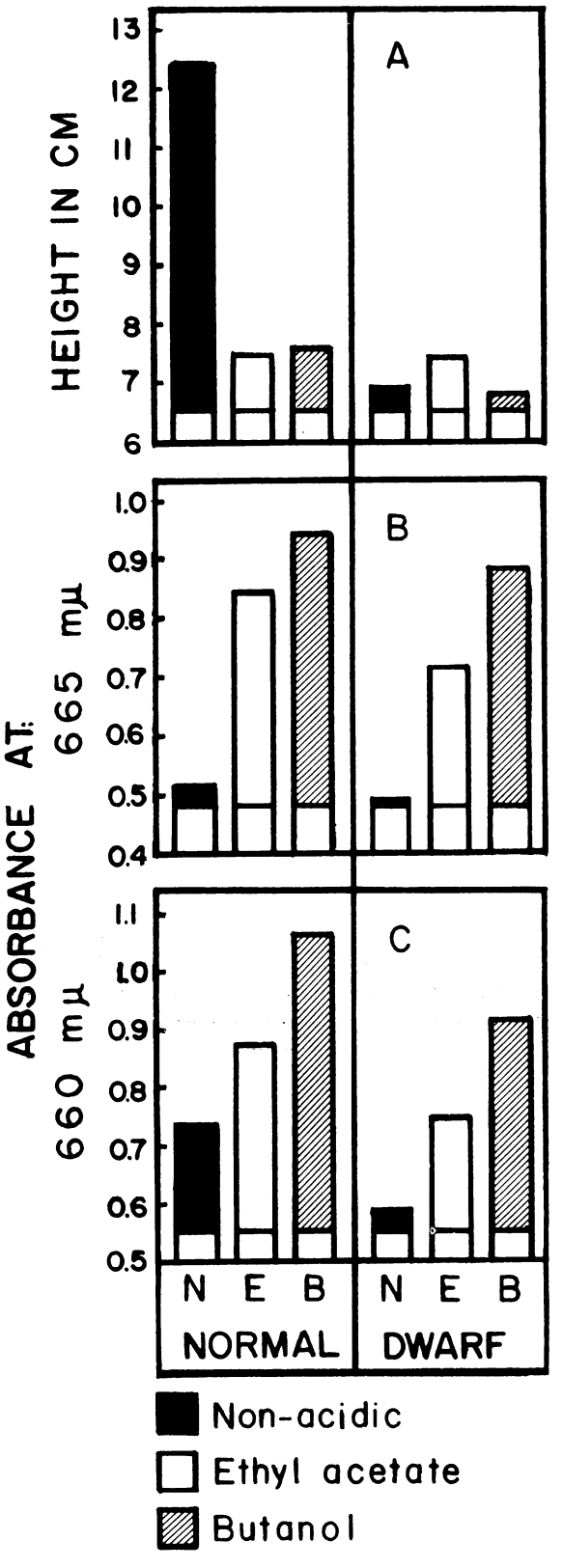

Table I. *Response Given to Crude Ethyl Acetate Extracts from Normal and Dwarf-1 Stems and Leaves in 2 Different Bioassays*

Differences (based on 20 replications) are significant at the 95 % level. Statistical variation within treatments was less than 7 %.

Source of extract	*Rumex* test	Wheat test
	mg/l equivalents of GA_3	
Normal	1.0×10^{-2}	1.8×10^{-2}
Dwarf-1	1.0×10^{-4}	2.0×10^{-5}

and leaves are presented in table I. The dwarf plant extracts, although less active than those of the normal line, still show considerable gibberellin activity. When, however, the extracts were applied to dwarf bean plants, no response could be detected, possibly because the hormones in the extracts were at a concentration below the sensitivity of the assay. When mature seeds were incubated with the various fractions and then germinated, those treated with the non-acidic fraction produced plants which were taller than the non-treated dwarfs.

Chromatography. Several differences were observed in the distribution of presumed gibberellins (*i.e.*, those fluorescent spots appearing only after treatment with sulfuric acid and heat) in the mature seed extracts. The acidic butanol fraction yielded 6 spots for the normal and only 3 for the dwarf. The acidic ethyl acetate fraction showed 5 and 4 spots, respectively, while no differences could be observed between the 2 lines in the non-acidic fractions since both had 2 presumed gibberellins at identical R_F values. A large fluorescent spot, however, was observed at R_F 0.9 prior to acid treatment in the normal non-acidic but not in the dwarf non-acidic fraction.

Rumex *and Wheat Endosperm Bioassays.* The results obtained in the *Rumex* and wheat seed bioassays compared with those of the Dwarf-1 bean test, reveal the inherent danger of relying on a single bioassay procedure when examining a range of gibberellin-like substances. In fact, both the *Rumex* and wheat test may also react to other materials such as the kinins. Both of the standard procedures used here indicated that there was more

Fig. 3. Results obtained in 3 different bioassays in response to the non-acidic, acidic ethyl acetate and acidic butanol fractions of extracts of seeds of the normal and Dwarf-1 lines. The results are shown for a typical experiment run with a single batch of seeds. Replicate experiments gave essentially identical results. The clear spaces at the bottom of the bars show the level of the controls. A) Dwarf-1 bioassay. B) *Rumex* bioassay. C) Wheat endosperm bioassay.

active material in the acidic butanol fraction than that extracted in the acidic ethyl acetate, while little or no activity was demonstrable in the non-acidic fraction. These results agree with those of Hashimoto and Rappaport (4) who also worked with bean seed.

It appears that there is no complete lack of gibberellin-like substances in the acidic butanol and acidic ethyl acetate fractions of the dwarf bean seed, but rather that the dwarf possesses lesser amounts of these materials. The results of the chromatographic studies indicate that this lesser activity could possibly be due to the absence of several individual gibberellins. None of these deficiencies, however, seem to be directly connected with control of normal growth, since if the missing factors (in the form of acidic extracts of the normal plant) are applied to the dwarf mutant, it does not revert to normal size. Such an effect might also be the result of a lack of penetration, the presence of sub-liminal concentrations of growth substances in the extracts, or a loss of sensitivity to the acid gibberellins as has been observed by Kende and Lang (5).

Loss of Leaf Pigmentation. A secondary effect of gibberellic acid on the Dwarf-1 mutant is the rapid loss of the green color of the leaves, often appearing 12 to 24 hours after treatment. This can be shown to be directly related to the dilution in chlorophyll content of a given leaf area by hormone induced expansion. The treated and expanded mutant leaf has a chlorophyll content per unit area identical to that of the normal, whereas the untreated dwarf leaf shows a dry weight and chlorophyll content 30 to 50 % greater than the normal (3). Wolf and Haber (16) concluded that a similar effect on dwarf wheat seedlings was also due to a dilution of the chlorophyll by gibberellin-induced growth.

A rapid loss in color in the Dwarf-1 mutant occurred 24 hours after application of the acidic ethyl acetate extract. The acidic butanol fraction induced no apparent changes, nor at first did the non-acidic fraction. After 96 hours chlorophyll dilution became apparent in the non-acidic fraction treated plants.

Conversion of Non-acidic Fraction Materials. The aforementioned delay in response is possibly a result of a time lag induced by conversion of non-acidic fraction materials into acidic ethyl acetate fraction substances which in turn induce the changes noted (4). Further evidence for such a conversion was presented by the behavior of seeds incubated with the various fractions. Only the non-acidic fraction of the normal seeds produced a response, and this too was delayed when compared with that of plants treated with gibberellic acid.

Basis of Response. The changes induced in the Dwarf-1 mutant after treatment with the non-acidic fraction may be explained by assuming that either the factors controlling internode elongation and leaf expansion are present in this fraction, or that the non-acidic fraction serves as a reservoir of precursors of these substances, which may be present in their ultimate form only in small amounts in actively growing plant organs.

If the latter is true, then bioassays based on isolated tissue systems such as the *Rumex* or wheat endosperm tests may be limited by having an absolute requirement for preformed gibberellins, whereas assays of the dwarf bean or dwarf corn type which employ the intact plant may be more suitable for precursor studies.

The findings of Chailakhyan and Sarkisova (1) that generative organs of seeded grapes contain larger amounts of gibberellins than the vegetative organs could serve to explain certain aspects in the developmental morphology of the Dwarf-1 mutant. The flowers, pods, and seeds of the dwarf bean are equal in size to the normal, and young seedlings, although smaller than normal seedlings of the same age, do undergo early elongation before slowing and stopping their growth at about the third internode. If there were reserve stores of hormones or hormone precursors in the seed, when these became exhausted elongation would be expected to cease. The inability to obtain a response with dwarf plants treated with normal plant stem and leaf tissue extracts which were able to elicit a reaction in the *Rumex* and wheat endosperm tests serves to reinforce this idea: the level of active material in mature tissue is probably quite low.

It may be concluded that dwarfism in this mutant results from a single-gene controlled metabolic block in formation of either gibberellin precursors or in non-acidic fraction gibberellin-like substances. Admittedly, this evidence is based mainly on seed extracts which may or may not be qualitatively the same as whole plant extracts, but the fact that dwarfism can be reversed by a normal plant extract, even though from seed, does lend credence to the argument.

Acknowledgment

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