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Rhizobial-Induced Increase in Internode Length and Identification of Endogenous GAs of Cowpea (*Vigna unguiculata* [L.] Walp) Stems and Nodules

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Abstract. Inoculation with *Bradyrhizobium sp.* strain 127E14 has been shown to cause a dramatic increase in the internode length of lima bean (*Phaseolus lunatus* L.), when compared to control plants inoculated with strain 127E15. This rhizobial-induced growth also occurs in cowpea (*Vigna unguiculata* [L.] Walp), an alternate host for the symbiont. Cowpea plants inoculated with strain 127E14 were 23% taller than those inoculated with strain 127E14 after 6 weeks of growth. Petiole length was found to be significantly greater in plants inoculated with strain 127E14. Cowpea plants treated at the apex with exogenous GA_3 or $GA_{4/7}$ responded by increasing internode length when compared to controls. As in lima beans, the rhizobial-induced growth response observed in cowpeas may be in response to an imbalance in the levels of GA-like substances within the plants. Gibberellins A_1, A_3, A_8, A_{19}, A_{20}, A_{29}, and A_{44} have been identified by GC-MS analysis in stems of cowpea, whereas the gibberellins A_1, A_{19}, A_{20}, A_{29}, and A_{44} were found to be present in nodule tissue formed by strain 127E14. The presence of these GAs indicates that the early 13-hydroxylation biosynthetic pathway is operative in cowpea. GAs identified in cowpea nodules are similar to those found in lima bean nodules formed by the same rhizobia. The finding that rhizobial strain 127E14 induces GA-dependent growth responses in two host legumes further supports the hypothesis that the presence of this bacteria alters the GA balance within the plant.

Phytohormones are known to regulate a diverse range of developmental processes in plants. In legumes, a very specialized form of development occurs when the soil bacterium *Rhizobium* enters into a symbiotic association with the plant. Each of the five classes of phytohormones has previously been implicated to function as a mediator in the process of nodule initiation (Grobbelaar et al. 1971, Phillips 1971a,b, Radley 1961, Thimann 1936). Far fewer studies have investigated the role of phytohormones in maintaining and controlling the symbiosis once it has been established. (Bouma 1970, El-Sherbeeney et al. 1977, Upadhyaya et al. 1991).

GA[1]-like substances have been found to be present in nodules formed on a number of leguminous plants (Atzorn et al. 1988, Dangar and Basu 1987, Dullaart and Duba 1970, Evensen and Blevins 1981, Radley 1961, Williams and DeMallorca 1982). Levels of GA-like substances are generally higher in nodules than in nearby root tissue (Evensen and Blevins 1981, Radley 1961, Williams and DeMallorca 1982). The abundance of GAs in the nodule has led to the hypothesis that rhizobia contribute directly or indirectly to the GA pool within this organ. The detection of GA-like substances in *Rhizobium/Bradyrhizobium* culture supernatants (Katznelson and Cole 1965, Lluch et al. 1983, Williams and DeMallorca 1982), argues for a direct

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[1] **Abbreviations:** BSTFA, bis-trimethyl-silyltrifluoroacteamide; DAP, days after planting; DW, dry weight; strain 127E14; strain 127E15, E15; EtOAc, ethyl acetate; EtOH, ethanol; GA_x, gibberellin A_x; FW, fresh weight; GC-MS, gas chromatography–mass spectrometry; KRI, Kovats retention index; MeOH, methanol; MeTMSi, methyl ester trimethylsilyl ether; PVPP, polyvinylpoly-pyrrolidone; SiO_2, silica gel; TMCS, trimethyl chlorosilane.

role for the rhizobial symbiont in determining nodule GA levels. Recent evidence that free-living *Rhizobium phaseoli* cultures produce GA_1 and GA_4 (Atzorn et al. 1988), supports the notion that rhizobia/bradyrhizobia have the genetic and metabolic capacity to synthesize GAs.

Using immunoassay, Atzorn et al. (1988) have found that the apparent GA content and composition of nodules are similar to that of cultured *Rhizobium* and uninoculated root tissue. These findings led them to conclude that the bacteria did not contribute significantly to the amount of GA within the nodule (Atzorn et al. 1988). Nodules formed on lima bean (*Phaseolus lunatus* L.) cv Henderson by *Bradyrhizobium sp.* strain 127E14 have been shown to contain relatively high levels of GA-like substances when compared to similar nodules formed by strain 127E15 or to nearby root tissue (Evensen and Blevins 1981). Thus, in contrast to the symbiotic system used by Atzorn et al. (1988), it appears that content of GA-like substances in lima bean nodules is influenced by rhizobial strain. To support this bioassay-based observation of Evensen and Blevins (1981), we have analyzed nodule extracts by GC-MS. Nodules formed on lima bean have been found to contain GA_1, GA_3, GA_{19}, GA_{20}, and GA_{44} (Dobert et al 1992). A similar range of 13-hydroxylated GAs were identified in lima bean stems (Dobert et al. 1992). Lima beans inoculated with strain 127E14, but not with strain 127E15 or other bradyrhizobia, also exhibited a marked period of rapid internode elongation (Evensen and Blevins 1981, Triplett et al. 1981). It is not known if the observed internode elongation is in response to the elevated levels of GA-like substances in the nodule.

Manhart and Wong (1980) originally utilized *Bradyrhizobium* strains 127E14 (−cNR) and 127E15 (+cNR) to determine the effect of constitutive nitrate reductase (cNR) activity on nitrogen fixation. These bradyrhizobia, originally isolated from lima bean nodules (Manhart and Wong, 1980), have also been found to form effective nodules on an alternate host, cowpea (*Vigna unguiculata* [L.] Walp). The promiscuous nature of these bacteria, nodulating two distinct legume species, provides the opportunity to study the role of the host plant in determining the quantity and quality of GAs in the nodule.

The role of GAs in mediating epicotyl elongation in cowpea (also called *V. sinensis* L.) has been extensively studied by Garcia-Martinez and Rappaport (1982, 1984, 1987). Their work has indicated that elongation of excised epicotyl segments in cowpea seedlings is mediated by the phytochrome system, in response to GAs supplied from primary leaves. Gibberellin A_1 and GA_{20} were found to be endogenous components of a combined extract of epicotyls, petioles, and leaves (Garcia-Martinez et al. 1987). They have speculated that 3β-hydroxylation of leaf-produced GA_{20} to GA_1 within the epicotyl is stimulated by far-red light (Garcia-Martinez et al. 1987). A number of GAs from immature seeds of cowpea, usually a rich source of GAs, have also been identified (Adesomoju 1977). The GAs identified by Adesomoju (1977), including GA_4, GA_6, GA_8, GA_{17}, GA_{19}, and GA_{20} and those tentatively identified, GA_1, GA_5, and GA_{29}, are indicative of the early 13-hydroxylation biosynthetic pathway.

Although exogenous GA application has been shown to cause a dramatic increase in epicotyl elongation of cowpea seedlings (Garcia-Martinez and Rappaport 1984), effects of GA on internode length and total plant height are poorly characterized. Okelana and Adedipe (1982) have reported that foliar GA application had little effect of total plant height in greenhouse-grown cowpeas. When these relatively tall (170–470 cm) cowpea varieties were treated with a series of foliar applications of GA_3 (20–2000 μg/plant), plant height, leaf, stem, and root DW were all unaffected (Okelana and Adedipe 1982). In contrast to these results, other studies (Foda et al. 1973, Sethuraj 1965) have found that foliarly applied GA causes dramatic growth increases in short-statured cowpeas, while a tall, "trailing" variety was similar in height to untreated controls.

It was our goal in this study to determine if the rhizobial-induced growth response that had been observed in lima bean (Triplett et al. 1981), also occurs in cowpea. Stem growth promotion observed in lima bean inoculated with strain 127E14 is presumed to be mediated by GAs (Evensen and Blevins 1981). Thus, since the effect of GAs on cowpea height remains equivocal, it was first necessary to determine if a short-statured cowpea line would respond to exogenous GA application. Further GC-MS characterization of endogenous GAs in vegetative tissue of cowpea was conducted to better understand the role of GAs in regulating stem elongation in this species. Likewise, the identification of endogenous GAs in nodules of cowpea, when compared to those GAs identified in lima bean nodules, will enable us to evaluate if a plant × *Bradyrhizobium* interaction occurs in determining GA status within the nodule.

Materials and Methods

Plant Material

Cowpea (*Vigna unguiculata* [L.] Walp.) seeds (cv. Purple Hull, Burpee Seed Co., Warminster, PA, USA) were soaked in a 10% EtOH solution for 3 min, rinsed, and germinated for 4 days on paper towels. Six seedlings per pot were planted in 20-cm pots filled with a 50:50 perlite:steam-sterilized sand mix. At the time of planting each seedling was dip-inoculated in an aqueous suspension of *Bradyrhizobium sp.* strain 127E14 or strain 127E15 (Nitragin, Milwaukee, WI, USA) derived from bacteria maintained and subcultured every month on solid yeast–mannitol medium (Vincent 1970). These rhizobial strains will hereafter be referred to as E14 (127E14) and E15 (127E15). Plants for the time course experiment were grown in a greenhouse without supplemental lighting (photoperiod in August approximately 13.5 h/day), and received a standard −N nutrient solution (Dobert et al. 1992) via a wick connecting the top pot with the lower nutrient reservoir. For growth experiments, 21-day-old plants were treated at the apex with 5 μl of a 10% ethanol solution containing either 0.5 μg GA_3 (Sigma) or $GA_{4/7}$ (Abbott Laboratories, courtesy of Dr. A. Cravetti). Control plants were treated with 5 μl of 10% EtOH. Following GA treatment, total plant height was measured at intervals of approximately 7 days until 42 DAP, when plants were harvested and plant dry weight, nodule number, and nodule mass were determined. In a second experiment, cowpeas were grown in the greenhouse as described above (photoperiod in May approximately 13 h/day). In these experiments a combination of fluorescent lights and incandescent lights (200 μE m^{-2} s^{-1}) were utilized to provide a photoperiod of 15 h/day. After 41 days of growth, internode and petiole length were determined and shoots and nodules were harvested.

Plants to be utilized for GA analysis were grown without supplemental lighting as indicated above; however, only strain E14 was used as inoculum. After 5 weeks of growth in the greenhouse, stem tissue above the first node and root nodules were harvested from each plant.

Extraction of GA-Like Substances

Nodule and stem tissue were frozen in liquid N_2 at time of harvest, and freeze-dried prior to analysis. A total of 18 g DW of nodules and 5 g DW of stems were used for analysis. Prior to any partitioning, [^{3}H]GA standards (approximately 600 KBq each of [^{3}H] GA_1 and [^{3}H]GA_4, Amersham) were added to MeOH extracts to provide an indication of losses during sample purification, and also to provide retention times during chromatographic separations. Extraction and partitioning procedures for nodule and stem samples have previously been described (Dobert et al. 1992). The resulting acidic, ethyl acetate extracts were loaded onto glass-fiber filter discs for SiO_2 column purification.

SiO_2 Partition Column Chromatography

Glass-fiber discs were placed on a SiO_2 column bed (Woelm 32-100, Universal Scientific, Atlanta, GA, USA), and eluted with a step gradient of formic acid-saturated hexane and EtOAc (Dobert et al. 1992, Durley et al. 1972). Stem extracts were eluted with five bed volumes of 50:50 hexane:EtOAc followed by seven volumes of 5:95 hexane:EtOAc and final MeOH wash. A three-step gradient was used for nodule extracts, consisting of five bed volumes of 70:30, 50:50, and 5:95 hexane:EtOAc and a MeOH wash in sequential order. Aliquots of the resulting fractions, each approximately one bed volume, were assayed for radioactivity. A single fraction for further analysis was produced by grouping all SiO_2 fractions lying between, and inclusive of, the peaks of [^{3}H]GA_4 and [^{3}H]GA_1.

Reverse-Phase HPLC Separation

The dried, grouped SiO_2 fractions were dissolved in MeOH and passed through a prewetted C_{18} cartridge Sep-pak and then filtered (0.45 μm Millex-HV, Millipore, Bedford, MA, USA). Samples were redissolved in 80% MeOH and subjected to reverse-phase C_{18} HPLC separation using a MeOH:acetic acid gradient (Dobert et al. 1992, Rood et al. 1986). Flow rate through the column (μBondapak C_{18}, 3.9 × 30 mm, Waters, Milford, MA, USA) was 1 ml/min. Forty-two fractions (2 ml/fraction) were collected, and aliquots assayed for radioactivity using liquid scintillation counting. To further analyze putative GAs, HPLC fractions 13–42 were grouped into seven pooled fractions (Fr1–Fr7), based on the radioactive profile of eluted [^{3}H]GA_1 and [^{3}H]GA_4.

Combined GC-MS Analysis

The dried, grouped HPLC fractions were redissolved in MeOH, and methylated for 1 h with ethereal diazomethane. Trimethylsilyl derivatives were produced by incubating samples for 30 min at 60°C in 50:50 BSTFA with 1% TMCS: pyridine (Rood et al. 1986). Derivatized samples, along with a hydrocarbon standard, to obtain KRI values (Gaskin et al. 1971) were co-injected onto a 15m DB-5 microcapillary column (J&W Scientific Inc., Folson, CA, USA) in a Hewlett-Packard 5890 Series II gas chromatograph, coupled to a Hewlett-Packard 5970A mass selective detector, programmed and operating as previously described (Dobert et al. 1992, Rood et al. 1986). Identification of putative GAs were made by utilizing the relative ion abundances from both selective ion monitoring (SIM), as well as full mass spectral scans for compounds eluting at KRI values corresponding to authentic GAs. Authentic standards were provided by Professors Richard Pharis (University of Calgary, Canada) and Lewis Mander (Australian National University, Australia).

Results

Cowpeas inoculated with *Bradyrhizobium sp.* strain E14 were significantly taller than those plants inoculated with strain E15 (Tables 1 and 3). In two separate series of experiments, it was found that total plant height after 6 weeks of growth was 22–26% greater for plants inoculated with strain E14 compared to those inoculated with strain E15. This in-

Table 1. Plant parameters from cowpeas inoculated with *Bradyrhizobium sp.* strain 127E14 or 127E15. Experiment 1.

	Rhizobial inoculum	
Parameter	127E14	127E15
Plant height (cm)	37.4 ± 4.6[a]	29.8 ± 1.89[a]
Plant DW (g)	3.79 ± 0.32	4.07 ± 0.36
Nodule DW (mg)	391 ± 38[b]	565 ± 49[b]
Nodule number	57.5 ± 7.0	61.4 ± 6.0

Values represent the mean of 12 plants harvested 42 days after planting ±SE.
Values significantly different at $p < 0.05$ ([b]) or $p < 0.10$ ([a]).

crease in height was minor compared to the differential growth response observed in lima beans (113%) inoculated with rhizobial strain E14 (Triplett et al. 1981).

Rhizobial-Induced Stem Growth

In the first set of plant growth experiments, plant height was measured at approximately 7-day intervals starting at 21 DAP. Differences in plant height due to rhizobial inoculum source were noticeable and significant only beyond 37 DAP (see Fig. 1). At 42 DAP, all plants were harvested and shoot mass, nodule mass, and nodule number were determined. Rhizobial strain was found to have a significant effect on total plant height and nodule DW, although nodule number was not affected (Table 1). The increase in plant height with E14 (26%) was not accompanied by any significant difference in shoot DW. The underlying cause for the difference noted in nodule mass between plants inoculated with E14 (391 g DW) and E15 (565 g DW) is not known. However, we have repeatedly observed that nodules formed on both cowpea and lima bean (Triplett et al. 1981) by strain E15 are generally larger than those resulting from E14 inoculation.

Although correlations cannot be used to show cause and effect, it is interesting to note that total plant height in plants inoculated with strain E14 was significantly correlated to nodule number (r = 0.87), nodule DW (r = 0.62), and nodule FW (r = 0.74) (Table 2). Such correlations between height and nodule number and mass did not exist for plants inoculated with strain E15. Although total nitrogen in plants was not determined, evidence from previous lima bean experiments (Triplett et al. 1981) suggests that the observed differences in cowpea height cannot be attributed solely to a variation in nitrogen supply due to rhizobial strain. Before assuming that the growth response observed in cowpeas inoculated with strain E14 was mediated by GAs, it was

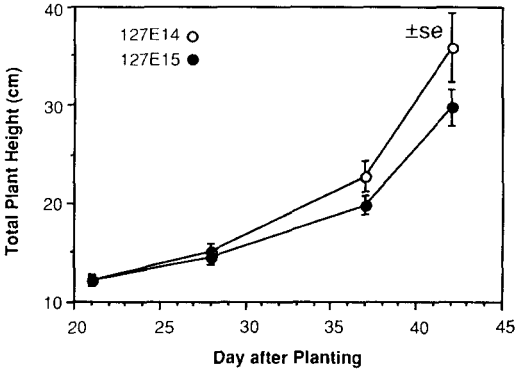

Fig. 1. Plant height of cowpeas inoculated with *Bradyrhizobium sp.* strain 127E14 or 127E15. Values represent mean (±SE) of 12 plants measured at weekly intervals.

Table 2. Correlation coefficients between plant height and nodule parameters for cowpeas inoculated with *Bradyrhizobium sp.* strain 127E14 or 127E15. Experiment 1.

	Rhizobial inoculum	
	127E14	127E15
Comparison	r	r
Plant height vs nodule DW	0.62[a]	0.18
Plant height vs nodule FW	0.74[a]	0.19
Plant height vs nodule number	0.87[a]	0.08

Correlation values based on 12 plants.
[a] Values significantly different at $p < 0.05$.

first necessary to determine the varietal response to exogenous GA application.

Effect of Exogenous GA Application on Cowpea Growth

Cowpea plants, inoculated with either strain E14 or E15, were treated at the apex with 0.5 μg GA_3 or $GA_{4/7}$ at 21 DAP. Internode length of cowpeas treated with either GA was significantly greater than untreated plants within 7 days (Fig. 2). Although only a single GA treatment was given to each plant, the relative difference between control and treated cowpeas continued to increase through the last sampling at 42 DAP. At the time of GA application most plants had approximately two expanded internodes, whose length did not increase further due to GA treatment. Exogenous GA addition created large differences in length of the third internode and all subsequent internodes (data not shown). Although treatment with GA_3 or $GA_{4/7}$ had dramatic effects on plant height, plant DW was ei-

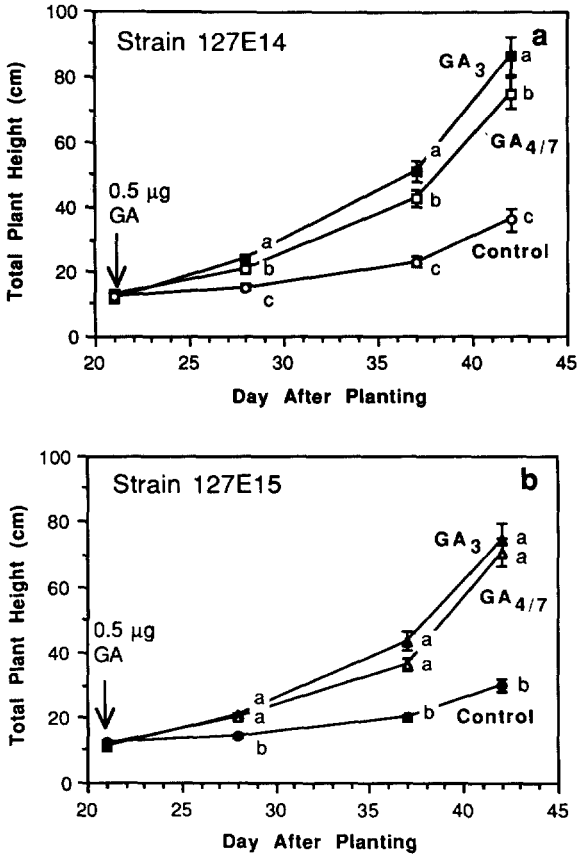

Fig. 2. Effect of GA_3 and $GA_{4/7}$ treatment on height of cowpeas inoculated with *Bradyrhizobium sp.* strains 127E14 or 127E15. Values represent mean (±SE) of 12 plants measured at weekly intervals. For each harvest date, points followed by different letters are significantly different at $p < 0.05$.

ther unaffected or only slightly elevated (data not shown).

Contrary to earlier findings with tall cowpea varieties (Okelana and Adelipe 1982), the cv. Purple Hull responded to both GA_3 and $GA_{4/7}$ treatment by displaying a significant increase in total height. GA treatment to a short cowpea variety has previously been shown to increase height over threefold when compared to controls (Sethuraj 1965). In cowpeas inoculated with strain E14, GA_3-treated plants were clearly taller (130%) than control plants, although, plants treated with GA_3 were also significantly taller than those treated with $GA_{4/7}$ (101% greater than control). This difference in efficacy in promoting internode elongation between GA_3 and $GA_{4/7}$ was not observed in cowpeas nodulated by strain E15 (see Fig. 2a and b). Differences in biological activity between GA_3 and GA_4 or GA_7 have been noted in several bioassay systems (Crozier and Durley 1983).

Twenty-one days after GA_3 application, cowpeas

Table 3. Plant parameters from cowpeas inoculated with *Bradyrhizobium sp.* strain 127E14 or 127E15. Experiment II.

	Rhizobial inoculum	
Parameter	127E14	127E15
Plant height (cm)	27.9 ± 0.92[b]	22.8 ± 0.69[b]
Plant DW (g)	2.79 ± 0.12	2.67 ± 0.16
Number of internodes	7.6 ± 0.2[b]	6.9 ± 0.2[b]
Nodule DW (mg)	177 ± 9[c]	219 ± 15[c]
Petiole length, 6th node (cm)[a]	12.24 ± 0.25[b]	11.06 ± 0.34[b]
Petiole length, 5th node (cm)	14.05 ± 0.32[b]	12.48 ± 0.36[b]
Petiole length, 4th node (cm)	12.20 ± 0.30	11.68 ± 0.38

Values represent the mean of 53 (127E14) and 39 (127E15) plants ±SE for plants harvested 41 days after planting.
[a] Approximate petiole location, actual location based on relation to apex.
Values significantly different at $p < 0.01$ ([b]) or $p < 0.05$ ([c]).

inoculated with either strain E14 or E15 were, respectively, 49 and 44 cm taller than untreated controls (Figs. 2a and b). Thus, the absolute magnitude of GA-promoted stem elongation was similar in cowpeas inoculated with either of the two *Bradyrhizobium* strains. Similarly, cowpeas nodulated by either strain E14 or E15 and treated with $GA_{4/7}$ displayed little difference in the magnitude of GA-promoted growth (38 and 41 cm, respectively).

Direct comparisons in total height between E14- and E15-inoculated cowpeas treated with GAs provide a different perspective. Total height at all time points following GA_3 application was found to be significantly greater for cowpeas inoculated with strain E14 when compared to E15-inoculated plants (compare Fig. 2a and b). Regardless of the prominent elongation due to GA_3 treatment, strain E14 further stimulates internode elongation and overall plant height, as in control plants. From these results it would seem that a rhizobial-induced growth factor and GA_3 treatment were additive. In cowpeas treated with $GA_{4/7}$, differences in total height due to rhizobial strain were not significant.

Growth Distribution and Petiole Length

In initial experiments, cowpeas inoculated with strain E14 were taller and, additionally, seemed to have longer petioles than plants inoculated with strain E15. Previous work has shown that petiole growth in several species is affected by GAs (Metzger 1988, Rood et al. 1989). A second set of experiments was carried out (using a larger plant population) to determine the effect of rhizobial inoculum source on petiole length, and to more accurately determine where, within the plant, growth promotion was occurring.

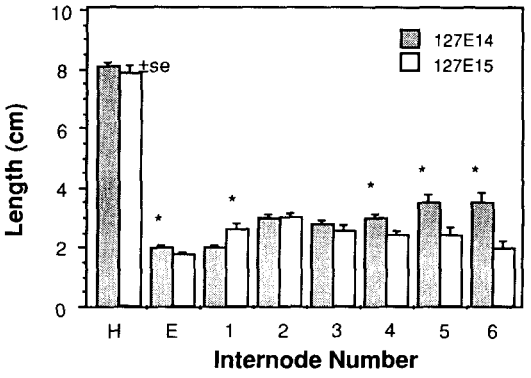

Fig. 3. Internode lengths of 41-day-old cowpeas inoculated with *Bradyrhizobium sp.* strain 127E14 or 127E15. Values represent mean (±SE) of 53 (127E14) and 39 (127E15) plants. On horizontal axis: H, hypocotyl length; E, epicotyl length. *Above columns indicates significant difference at $p < 0.05$.

As in the first experiment, total height of cowpeas inoculated with strain E14 was significantly greater than height of plants inoculated with strain E15 (Table 3). Similarly, shoot DW was unaffected by treatment, while the DW of nodules from cowpeas inoculated with strain E15 was again greater than the mass of nodules formed by strain E14 (Table 3). Total number of internodes (expanded and unexpanded) was also found to be greater (10%) in E14 plants when compared to E15 plants (Table 3). Although an increase in internode number alone could yield plants that varied in total height, it was determined that significant differences also existed in length of the epicotyl, and 1st, 4th, 5th, and 6th internodes (Fig. 3). For these determinations only, those internodes longer than 1 cm were included in comparisons. Length comparisons between the most apical internodes (7th or above, when present) were not made due to variability in their stage of development. While the epicotyl, 4th, 5th, and 6th internodes were all longer in plants inoculated with strain E14, length of the 1st internode was greater in those plants inoculated by E15 (Fig. 3). Thus, the increase in total height (23%) due to rhizobial strain is in part attributable to the increase in total internode number, while the remainder is due to a greater length of upper internodes.

Petiole length in cowpeas was found to be significantly greater in plants inoculated with strain E14 when compared to those inoculated with strain E15 (Table 3). Values were obtained by measuring the length of petioles supporting the three most recently expanded leaves. In most cases, these petioles were attached to nodes 4, 5, and 6. While differences in length due to rhizobial strain were significant for petioles occupying the most apical positions (i.e., those most often attached to nodes 5 and 6), length differences in the more basal petiole were insignificant (Table 3). Metzger (1988) has noted that only immature petioles are sensitive to exogenous GA application and respond by elongating. Thus, it is possible that levels of rhizobial-induced growth factors do not exceed a threshold value until after the 4th petiole has matured. Similarly, only internodes above the 4th node show a significant difference in length (Fig. 3) due to rhizobial strain.

GA Analysis

Gibberellins A_1, A_{19}, A_{20}, A_{29}, and A_{44} were identified in nodules formed on cowpea by *Bradyrhizobium sp.* strain E14 (Table 4). These identifications were based on similarities in HPLC and GC retention times and ion fragmentation pattern between authentic standards and putative GAs. A representative multiple ion trace from nodule extracts is shown in Fig. 4. The prominent peaks at 13.95 min correspond to fragment ions from GA_{20} MeTMSi derivatives. The presence of these 13-hydroxylated GAs is consistent with the notion that they are derived from the early 13-hydroxylation biosynthetic pathway. At this time it is not known if these compounds were synthesized within the nodule, or if they have been translocated from some distant site. Since the nodule is comprised of both bacterial and plant fractions, it is possible that the GAs identified are located solely in either, or in both compartments. Although not definitive, there was good evidence to suggest that GA_3 was also present in nodules, as indicated by a strong 504 amu peak at a KRI value of 2731. Extracts were further analyzed by SIM for GA_4, GA_7, GA_{17}, and GA_{53}; however, no evidence for their presence in nodules was obtained. Based on abundance of the major ion, and assuming equivalent recoveries, relative quantities of the GAs identified were $GA_{20} >> GA_{44} >> GA_{29} > GA_1 > GA_{19}$.

In purified stem extracts from 37-day-old cowpeas, GA_1, GA_3, GA_8, GA_{19}, GA_{20}, GA_{29}, and GA_{44} were identified (Table 4). The identity of these compounds was based on comparisons in chromatographic retention times, including KRI values, and ion fragmentation pattern between authentic GA standards and putative GAs in extracts. As in nodules, the GAs identified are indicative of the early 13-hydroxylation biosynthetic pathway, and suggest that this pathway predominates in cowpea stems. Another member of this pathway (GA_{53}), and several metabolic offshoots (GA_4, GA_5, GA_6, and GA_{17}) that have been identified in immature

Table 4. GC-SIM of MeTMSi derivatives of authentic GAs and putative GAs from cowpea nodules and stems.

Gibberellin—Source	HPLC fractions	KRI	Constituent ion (% abundance)				
Authentic GA_1	22–23	2703	506 (100)	491 (13)	448 (20)	377 (12)	313 (17)
Putative GA_1—Stem	22–25	2703	506 (100)	491 (10)	448 (29)	377 (34)	313 (20)
Putative GA_1—Nodule	22–25	2704	506 (100)	491 (12)	448 (19)	377 (10)	
Authentic GA_3	21–22	2732	504 (100)	489 (10)	370 (9)	347 (10)	208 (45)
Putative GA_3—Stem	22–25	2731	504 (100)	489 (9)	370 (16)	347 (19)	208 (118)
Authentic GA_8	12–14	2835	594 (100)	579 (7)	535 (10)	448 (60)	
Putative GA_8—Stem	13–18	2835	594 (100)	579 (9)	535 (7)	448 (56)	
Authentic GA_{19}	30–31	2651	462 (15)	434 (100)	402 (40)	375 (60)	
Putative GA_{19}—Stem	30–33	2650	462 (10)	434 (100)	402 (38)	375 (74)	
Putative GA_{19}—Nodule	30–33	2649	462 (9)	434 (100)	402 (34)	375 (50)	
Authentic GA_{20}	27–28	2536	418 (100)	403 (15)	375 (50)	359 (12)	301 (13)
Putative GA_{20}—Stem	26–29	2538	418 (100)	403 (14)	375 (63)	359 (16)	301 (15)
Putative GA_{20}—Nodule	26–29	2538	418 (100)	403 (15)	375 (51)	359 (12)	301 (20)
Authentic GA_{29}	13–17	2707	506 (100)	491 (10)	389 (10)	375 (15)	303 (17)
Putative GA_{29}—Stem	13–18	2704	506 (100)	491 (10)	389 (6)	375 (26)	
Putative GA_{29}—Nodule	13–18	2705	506 (100)	491 (9)	389 (4)	375 (16)	303 (37)
Authentic GA_{44}	28–29	2850	432 (65)	417 (15)	373 (20)	238 (41)	207 (100)
Putative GA_{44}—Stem	30–33	2850	432 (65)	417 (15)	373 (21)	238 (38)	
Putative GA_{44}—Nodule	30–33	2850	432 (50)	417 (13)	373 (14)	238 (24)	207 (100)

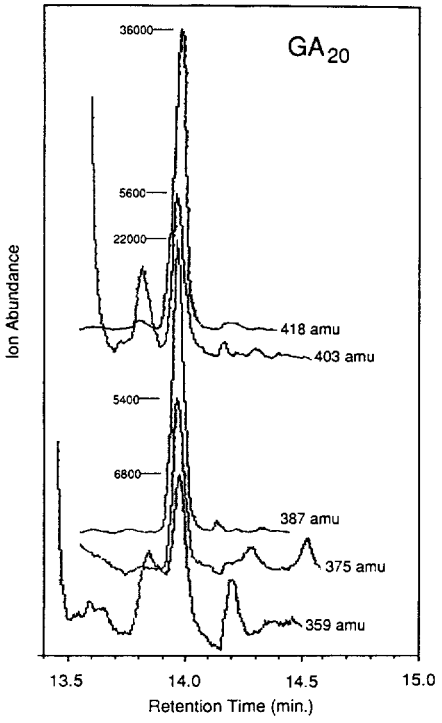

Fig. 4. SIM traces of characteristic fragment ions of MeTMSi derivatives of GA_{20} from cowpea nodule extracts. Nodules from plants inoculated with *Bradyrhizobium sp.* strain 127E14 were harvested 5 weeks after planting. Nodules extracts were purified by SiO_2 and HPLC and analyzed by GC-MS as described in Materials and Methods.

seeds (Adesomoju 1977) were not detected in the extracts analyzed. Based on the abundance of the major ion, and again assuming equal recoveries, GA levels in the stem were generally lower than those in the nodule.

Discussion

Lima beans inoculated with *Bradyrhizobium sp.* strain E14 were significantly taller than plants inoculated with strain E15 (Evensen and Blevins 1981, Triplett et al. 1981). Findings that exogenous GA application mimics the rhizobial-induced growth response, while GA biosynthesis inhibitors suppress the phenotype, led to the conclusion (Evensen and Blevins 1981, Triplett et al. 1981) that the phenomenon was mediated by GAs. Thus, one of the objectives of this study was to determine if a rhizobial-induced growth response occurred in cowpea, an alternate host for this bacteria. A preliminary study (Heitholt 1980) indicated that rhizobial-induced height differences were not significant in cowpea. Although the differences in total height between cowpeas inoculated with strains E14 and E15 were not as dramatic as that observed in lima bean (Triplett et al. 1981), they were, nonetheless, significant at 37 DAP (Fig. 1) and were observed in plants grown under two different light regimes.

While it is possible that differences in height due to rhizobial strain are the result of a variation in

nitrogen supply from the nodules, several lines of evidence argue against such a mechanism. Research suggests that an increased supply of symbiotically fixed nitrogen results in a general stimulation of plant growth, which results in a greater shoot weight (El-Sherbeeney et al. 1977). Cowpeas inoculated with strain E14, however, have a shoot biomass equivalent to those plants inoculated with strain E15 (Tables 1 and 3). Secondly, our data indicate that the weight of nodules formed on cowpea by strain E15 is greater than nodules formed by strain E14 (Tables 1 and 3). Thus, if nodule weight is loosely related to N_2-fixation potential, these results refute the notion that plants inoculated with strain E14 are receiving more fixed nitrogen. Furthermore, Triplett et al. (1981) have found that lima beans inoculated with these two strains show no significant difference in either N content or acetylene reduction rates.

Cowpeas (cv Purple Hull) treated with GA_4 or $GA_{4/7}$ responded rapidly and dramatically with increased total plant height (Fig. 2a and b). Foliar GA application to several tall cowpea cultivars (Adzuki, Ife Brown, and New Era) had previously been found to have no effect on plant height (Okelana and Adedipe 1982). The discrepancy in results is most likely due to the differences in plant morphology, since tall plants, when compared to those that are short-statured, are often less responsive to exogenous GA application (Sethuraj 1965, personal observation in pole lima beans). In support of this hypothesis, Sethuraj (1965) has found that foliar application of 50 μg/ml of GA_3 caused a threefold increase in height of a bushy variety (ECR 1548) of cowpea, while a "trailing" variety (EC 455) showed no response.

The relative increase in plant height brought about by 0.5 μg GA_3 treatment to cowpea (130%) was nearly as large as that observed in lima beans (170%) treated with 10 times more (5.0 μg) GA_3 (Evensen and Blevins 1981). A dose–response experiment would be necessary to determine if the sensitivity to exogenous GAs differs between lima beans and cowpeas. Such an experiment, together with the determination of endogenous GA content, may help determine the basis of the considerable difference in the magnitude of the growth response observed between cowpeas and lima beans inoculated with strain E14.

As an isomeric analog of the biologically active GA_1, it is not surprising to note that GA_3 is more effective than $GA_{4/7}$ in promoting internode elongation (Fig. 2a). If, like some other members of the *Leguminosae,* internode length is primarily regulated by GA_1 (Ingram et al. 1984), GA_4 or GA_7 would be inherently inactive prior to 13-hydroxylation to GA_1 or GA_3 within the plant. Additionally, the finding that GA_3 is more effective than $GA_{4/7}$ in promoting internode elongation in cowpeas inoculated with strain E14, but not strain E15, may indicate that GA sensitivity or metabolism differs within the two symbiotic systems. A role for the rhizobial strain in determining sensitivity to exogenous GAs has been postulated for lima beans (Evensen and Blevins 1981). In lima beans inoculated with E15, Evensen and Blevins (1981) found that the magnitude of the growth response to GA_3 decreased as plants matured, while responsiveness was maintained in plants inoculated with strain E14. They speculated that a failure to release GA synergists, or overabundance of GA antagonist could decrease responsiveness in E15 plants (Evensen and Blevins 1981). GA application and rhizobial-induced growth were found to be additive effects in cowpea. Thus, as in untreated plants, GA-treated cowpeas inoculated with E14 were taller than E15 plants (Fig. 2a and b). A similar additive response between GAs and rhizobial-induced growth has been observed in lima bean (Evensen and Blevins 1981).

The rhizobial-induced increase in total height could be attributed to a combination of factors. Cowpeas inoculated with strain E14, when compared to E15 plants, possessed a greater number of internodes, and upper internodes (4th through 6th) were found to be greater in length (Fig. 3). Similarly, the increase in total height in lima beans inoculated with E14, when compared to E15-inoculated plants, was the result of an increase in length of internodes 3 through 7 (Triplett et al. 1981).

Differences in length of the epicotyl and the first internode between plants inoculated with the different rhizobia (Fig. 3), are noteworthy since this stem tissue develops and matures during the initial stages of the symbiosis. Garcia-Martinez and Rappaport (1982) have shown that epicotyl elongation in cowpea was completed by 11 to 12 DAP, and our studies have indicated that exogenous GA application at 21 DAP did not promote further elongation of the epicotyl (data not shown). Thus, it would seem likely that differences in epicotyl length between E14- and E15-inoculated plants were manifested early in development, and did not occur at some latter time. The means by which root nodule bacteria can bring about this change in plant growth at such an early time is not known. It is possible that even before a macroscopic nodule develops (approximately 12–14 days), invading E14 bacteria can alter the GA balance within the plant, resulting in a greater final length in the highly sensitive epicotyl tissue. The fact that the first internode of E15 plants is longer than that of E14 plants, may be attributed

to the excessive epicotyl elongation in E14 plants. Diversion of available photosynthate, produced largely by primary leaves, to the expanding epicotyl of E14 plants might subsequently limit 1st internode growth.

In a number of species, petiole length appears to be regulated by GAs. Rood et al. (1989) have found that petiole length is greatly diminished in a GA-deficient line of *Brassica*. Conversely, when white clover (*Trifolium repens* L.) is treated with GA_3 petiole length is significantly greater than in untreated controls (Norris 1989). Metzger (1988) found that petiole length in *Thlaspi arvense* was independently regulated by both GAs and photoperiod. He determined that petiole growth was sensitive to exogenous GA application only during the early stages of its development, whereas photoperiod could influence petiole growth regardless of developmental state. In cowpea the length of the most distal petioles was significantly increased by inoculation with strain E14 (Table 3). Since petiole length was not measured in GA-treated cowpeas, it can only be speculated that the observed increase in petiole length is mediated by GAs. The observation that rhizobial-induced increases in the length of petioles near the apex (relative positions 5 and 6) corresponded with an increase in the length of nearby internodes (4th, 5th, and 6th) suggests that these two processes may be regulated by a single factor. As in lima bean (Evensen and Blevins 1981), we suggest that the observed growth response in cowpeas inoculated with strain E14 is mediated by GAs.

In lima bean nodules formed by strain E14, a number of GAs characteristic of the early 13-hydroxylation pathway have been identified (Dobert et al. 1992). Similarities in the range of GAs between stem and nodule tissue failed to provide firm evidence that the rhizobial strain makes a qualitative contribution to the GA pool in either the nodule or the shoot.

Using cowpeas inoculated with the same rhizobial bacteria, the role of the host plant in determining the spectra of GAs present within the nodule might be determined. Characterization of GAs present in vegetative stem tissue would facilitate an understanding of how GAs regulate growth in cowpea. In cowpea nodule extracts, GA_1, GA_{19}, GA_{20}, GA_{29}, and GA_{44} were identified (Table 4). These 13-hydroxylated GAs are identical to those identified in lima bean nodules (Dobert et al. 1992). In cowpea stems, many of the same GAs have been identified (Table 4). Only GA_8, the biologically inactive, 2β-hydroxylated metabolite of GA_1, is unique to stem tissue. The finding of these GAs in stem tissue, when compared to those identified by Adesomoju (1977) in seeds, seems to indicate that similar biosynthetic pathways are operational in both reproductive and vegetative tissues. In epicotyls, where GA_1 and GA_{20} have been identified, it is speculated that elongation is regulated by GA_1 (Garcia-Martinez et al. 1987). These GAs were also present in extracts of older stems, and, thus, it is possible that elongations in this tissue is under the influence of GA_1.

The similarity of GAs in cowpea and lima bean stems limits our ability to determine the effect of the host plant on GA status of the nodule. Likewise, since a significant difference in the range of GAs present was not apparent in either stems of the host plant or nodules on these plants, we cannot fully assess the contribution of the rhizobial microsymbiont to the GA makeup of the nodule. We are currently investigating if different *Bradyrhizobium* strains have a quantitative impact on GA status of the nodule. The identification of GAs in nodules and stems of cowpeas was necessary to determine the means by which *Bradyrhizobium sp.* strain E14 promotes internode elongation in this host plant. The finding that this bacteria can stimulate elongative growth in two different hosts, further strengthens the argument that these changes are attributable to an alteration in GA level or responsiveness.

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