Effect of rhizobitoxine on growth attributes, yield and some biochemical parameters of soybean

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ABSTRACT

Rhizobitoxine –producing (RT^+) strains of *Bradyrhizobium japonicum*, differing in their abilities to induce folair chlorosis with soybean (*Glycine max* [L.] Merr.) variety Giza 82, were evaluated for effects on short term shoot productivity, nodulation, N_2 fixation, and nodule protein production under greenhouse conditions. Soybeans were singly inoculated with washed suspensions of (Group II) USDA strains 38, 138, 142, 191, 110, 206 or 217. Strains USDA 110 and USDA 206 (Group I/Ia) were included as RT-controls. The plants were cultured in the absence of combined N in horticultural- grade vermiculite for 50 days. Beginning 22 days after planting, plants were evaluated weekly for chlorophyll, leaf protein and biomass accumulation, nodular contents of leghemoglobin, soluble protein and RT, and total shoot N content. Rhizobitoxine was detected in nodules of all RT^+ strains with the exception of USDA 38. However, only USDA 142 and USDA 191 produced both quantifiable concentrations of RT and symptoms of RT – induced chlorosis. Coincident with moderate to severe chlorosis were reductions in chlorophyll concentrations, shoot and nodule dry weight, leaf protein and total N_2 fixation. During extended periods of severe chlorosis, reductions in Lb and soluble nodular protein were observed. Based on carbon accumulation, all non-chlorotic treatments were statistically more productive than the chlorotic treatments. Similarly, non-chlorotic Group II treatments tended to fix less carbon relative to the RT- Group I/Ia controls, although these differences were not statistically significant. The results of this study suggest that, in the absence of discernable foliar chlorosis, the effect of RT^+ (Group II) nodulation on short term soybean productivity is minimal.

Key word: Chlorophyll, leghemoglobin, *bradyrhizobium japonicum*, rhizobitoxine, nodule protein

INTRODUCTION

Rhizobitoxine (RT), a low molecular weight amino acid produced by some strains of *Bradyrhizobium japonicum*, has been identified as 2-amino-4-(2- amino-3-hydroxypropoxy)-trans-but-3-enoic acid (Owens *et al.*, 1972; Keith *et al.*, 1975). A number of studies have examined the expression of chlorosis in soybean nodulated by RT- producing (RT^+) *B. Japonicum* (Erdman *et al.*, 1956, 1957; Johnson *et al.*, 1958, 1959). Rhizobitoxine symptoms expressed in greenhouse-grown soybean generally appear as chlorosis in the meristematic regions of the plant accompanied by necrosis

of stem and petiole tissues. In less severe cases, irregular coloration of the leaf or light marginal chlorosis may appear.

The characterization of rhizobitoxine-producing (RT^+) strains of *Bradyrhizobium japonicum* has gained increased attention in recent years (Devine *et al.*, 1988; La Favre *et al.*, 1988; Fuhrmann, 1990; Minamisawa and Fukai, 1991). Strains of *B. Japonicum* can be divided into two major groups based on DNA homology (Hollis *et al.*, 1981; Stanley *et al.*, 1985; El-Sayed 1995 & 1998). In all cases studied, Group I/Ia and II strains have been shown to be RT^- and RT^+, respectively (Minamisawa, 1989; Minamisawa and Fukai, 1991; Abdel-Aziz *et al.*, 2003). However, not all Group II strains appear to induce the foliar chlorosis in soybeans associated with RT production, presumably as a result of differences in the levels of RT production among RT+ strains (Devine *et al.*, 1988; Fuhrmann, 1990; Minamisawa, 1990; Abdel-Mawly and El-Sayed, 1999).

RT-producing strains of *B. japonicum* can represent a significant component of indigenous soil populations. For example, Fuhrmann (1990) reported that 18% of the *B. japonicum* isolates sampled from Delaware farms induced visible rhizobitoxine symptoms in the greenhouse and that all of these appeared to belong to Group II. Overall, 37% of the isolates surveyed conformed to phenotypes corresponding to the Group II homology group.

The effect of nodulation by RT^+ *bradyrhizobia* on soybean productivity has received surprisingly little attention. A study by Erdman *et al.* (1957) found that the expression of severe RT symptoms among several soybean varieties nodulated by *B. japonicum* strain USDA142 resulted in decreased vegetative yield and pod weight. A study by Fuhrmann (1990) revealed that, in general, strains of *B. japonicum* from RT^+ genotypes (Group II strains) fixed less nitrogen than strains from RT^- genotypes (Group I/Ia) regardless of symptom severity.

Although many studies have examined soybean shoot response to nodulation by RT^+ *bradyrhizobia* (Erdman *et al.*, 1957; Fuhrmann, 1990; El-Sayed and Abdel-Mawly, 1999), the studies on nodule function are lacking. Rhizobitoxine can inhibit methionine and ethylene biosynthesis (Owens *et al.*, 1968, 1971; Giovanelli *et al.*, 1972; Mohamed and El-Sayed, 2003), suggesting that it may disrupt protein synthesis which is important to nodule activity. Soybeans grown under greenhouse conditions in the absence of combined N have been observed to exhibit accelerated nodule senescence when nodulated by chlorosis-inducing strains (RTC^+) of

bradyrhizobia. These reports suggest that RT- induced effects on the production and activity of symbiotically important proteins such as leghemoglobin may prove valuable in assessing the symbiotic response to RT^+ strains of *B. Japonicum*.

A side from the visible chlorosis caused by RT, there is little additional information in the literature concerning basic plant responses to the toxin. Considering the significant numbers of RT^+ strains present in soils of the southeastern and mid-Atlantic regions of the U.S. (Erdman *et al.*, 1957; Weber *et al.*, 1989; Fuhrmann, 1990; El-Sayed and Abo-El-Wafa 2001), the effect of RT on soybean productivity should be clarified in order to assess the impact of indigenous *B. japonicum* strains on soybean production. The purpose of this study was to characterize the plant response to nodulation by *B. japonicum* strains differing in levels of RT production. Specifically, the effect of RT^+ strains on nodulation, N_2 fixation, biomass accumulation, chlorophyll accumulation, and the production of nodule and leaf proteins.

MATERIALS AND METHODS

Inoculum preparation and greenhouse culture:

All *B. japonicum* strains used were obtained from Microbiology Research Center, Cairo Mircen, Egypt (EMCC), Fac. of Agric., Ain Shams Univ., Cairo, Egypt). The strains included USDA38, 138, 142, 191, 110, 206, and 217. USDA 110 and 206 are Group I/Ia strains whereas the remainder belong to Group II (Hollis *et al.*, 1981; Devine *et al.*, 1988). Strains USDA 191 and 142 are known to cause severe RT- induced chlorosis, whereas USDA 217 may induce moderate symptoms (Devine *et al.*, 1988). Strain USDA 38 may induce light RT symptoms (Erdman *et al.*, 1957), whereas USDA 138 is not documented to be a chlorosis-inducing strain. However, many strains within serogroup 138 are RTC^+ (Fuhrmann, 1990; Minamisawa, 1990). Strains USDA 110 and 206 were included as RT^- controls. Strain USDA 110 is a highly effective N_2-fixing strain and exhibits the hydrogenase phenotype (Hup^+) whereas USDA 206 is Hup^- and a moderately effective N_2 fixer (Fuhrmann, 1990; Minamisawa, 1990).

Strains of *B.japonicum* were cultured in a yeast extract-mannitol broth containing the following components (L^{-1}) : yeast extract (Difco), 0.5g; mannitol, 10g; Mg SO_4. 7 H_2o, 0.2g; Nacl, 0.1g; K_2 HpO_4, 0.5g (pH 7.0) (Weaver and Frederick, 1982). Bacterial cells were harvested at the late-logarithmic growth phase, centrifuged, washed and diluted with sterile distilled water to a density of approximately 10^6 cells mL^{-1}.

Soybean seeds (Giza 82) were surface disinfected with 95% ethanol for 5 min, rinsed with sterile distilled water, and sown to 600-mL pots containing horticultural grade vermiculite as a rooting medium (4 seeds pot^{-1}). Seeds were then inoculated with individual *B. japonicum* strains at a rate of 4 mL pot^{-1}. Noninoculated controls were also prepared. Seedlings were thinned to 2 per pot at 12 days after planting (DAP). Plants were watered as needed with *a* modified N-free nutrient solution (McClure and Israel, 1979) containing 6.5 *m* $CaSO_4$, 1.0m *M* KH_2PO_4, 0.5m *M* K_2HPO_4, 2.0m *M* $MgSO_4$, 17.8μ *M* Fe-EDTA, and micronutrients as described by Ahmed and Evans (1960). Pots were flushed with water one a week prior to application of nutrient solution to avoid excessive salt accumulation.

The experiment was installed using a randomized complete block design with 4 replications. Two pots were prepared for each sampling date-strain-rep combination (denoted below as pot 1 and pot 2) to allow sufficient plant material for analysis. Plants were destructively harvested at 22, 29, 36, 43 and 49 DAP.

Studied Characters

The following plant Characters were estimated according to the recommended methods.

(A) Shoot Analysis:

1- General characterization

At each harvest, plants were evaluated for the expression of RT symptoms. Symptoms were rated using a scale of 0 to 5, with 0 denoting no symptoms. Leaf surface area for pot 1 plants was measured using a Hayaski Denko automatic area meter, and associated fresh weights were obtained. Shoots from pot 2 plants were severed at the cotyledonary node, dried at 65°C and weighed.

2- Leaf protein and pigment assays

The center leaflet of each trifoliate from pot 1 plants was sampled, collectively weighed and ground in 80% acetone buffered with 0.05 *M* Tris-HCl (pH 7.0). Samples were maintained under subdued light and low temperature during processing to minimize pigment degradation. Leaf extracts were centrifuged at 10,000 Xg for 15 minutes, and the resulting supernatant collected. The pellets were resuspended in 2 *M* NaOH for 12

hours at 4°C. Samples were then recentrifuged, and the NaOH supernatants were collected.

Concentrations of chlorophyll a and b in the acetone extract were estimated spectrophotometerically as described by Arnon (1949). Soluble leaf protein in the acetone extract was estimated using a modified Folin technique as described by Peterson (1977). Bovine serum albumin was used as a standard. Acetone insoluble leaf protein concentrations in the NaOH extracts were measured in similar manner. Total leaf protein was calculated as the sum of soluble and insoluble protein estimates.

3- Kjeldahl assay

Dried (65°C) shoot material from pot 2 plants were ground in a Wiley Mill to pass a 20-mesh screen. Total reduced nitrogen was determined using a standard kjeldahl procedure (Bremner, 1965).

(B)Nodule Analyses

1- Leghemoglobin and nodule protein assay

Nodules from pot 1 roots were picked, rinsed in deionized water and weighed. Separation of the bacteroids from the nodule cytosol was achieved by homogenizing nodules at 4°C in a reduced phosphate buffer (25 m *M* sodium phosphate [pH 7.0], 1m *M* $Mgcl_2$, and 1m *M* dithioerythritol) followed by centrifugation at 10,000 Xg for 15 min (Pfeiffer *et al.*, 1983). Soluble protein content of the nodule cytosol (supernatant) was determined using a modified Folin protein assay described by Peterson (1977).

Leghemoglobin (Lb) content in the cytosol was determined using the pyridine hemochrome assay proposed by Bergersen *et al.* (1973). The reduction or oxidation of hemochrome samples was achieved with the addition of sodium dithionite or potassium hexacyanoferrate (III), respectively. The difference spectra were calculated as the reduced minus the oxidized spectra using a Hewlett Packard Diode Array Spectrophotometer model 8452a. Hemochrome concentrations were estimated from difference spectra at 556 and 539 nm using the equation described by Bergersen *et al.* (1973). Molar concentrations of Lb were converted to mg ml^{-1} using an average molecular weight of 16, 375 (Fuchsman and Appleby, 1979).

2- Acetylene reduction assay

Total nitrogenase activity was determined on excised, washed root systems using an acetylene reduction technique (Weaver and Frederick, 1982).

3- Rhizobitoxine assay

Rhizobitoxine content of pot 1 nodules was estimated using thin layer chromatography (TLC). Nodule cytosol was partially purified by loading onto an ammonium-saturated Dowex 50-X4 ion exchange column (200-400 mesh, pH 8.0 –9.0) and washing with 10 bed volumes of deionized water. Nodule samples were eluted with 1 *M* NH_4 OH and concentrated *in vacuo* to remove the ammonia (Minamisawa and Kume, 1987). Samples were resuspended in 50% methanol and applied to fibrous cellulose TLC plates (20 Cm X 20 Cm X 250 μm). At total of 10 μL was applied to each lane in 1 μl aliquots to ensure a compact application area. Plates were developed twice (with intermitten drying) to a distance of 16 Cm in butanol: acetic acid: water (4:1:1). The detection reagent (0.5% ninhydrine in 95% EtOH buffered with 5% collidine) was applied as an aerosol mist (Owens and Wright, 1965). Plates were heated for 3 minutes with a heat gun and scanned on a Shimadzu cs 9000 dual-wavelength flying-spot scanner at a wavelength of 440 nm. Rhizobitoxine was distinguished from other amino acids by its yellow reaction with ninhydrine and an average R_F of 0.18. Due to a lack of pure rhizobitoxine for use as a quantitative standard, toxin samples were quantified relative to asparagines (i.e., as asparagine equivalents [as n eqv.]) which produces a similarly colored reaction with ninhyrine. An impure qualitative RT standard, obtained using the methods of Owens and Wright (1965), was used to verify the location of the toxin on the plate. The detectable limit of the analysis ranged from 13 and 75 n mol as n eqv. $Plant^{-1}$, depending upon the particular analytical run, with an overall mean of 33.0 n mol. $Plant^{-1}$.

(C) Statistical Analyses

Regression analysis of the data was performed using the General Linear Model Procedure of the Statistical Analysis System (SAS Institute, Cary, NC, 1985). Analysis of variance suggested the presence of strain related heteroscedasticity (heterogeneity of variances) for all parameters expressed on a whole plant basis [e.g. chlorophyll content, shoot dry weight, Lb content, etc.). Variances associated with means of non-chlorotic

treatments were significantly greater than those associated with chlorotic treatments. Therefore, Least Significant Difference Values were conservatively calculated for all affected parameters by excluding the chlorotic treatments from the statistical analysis (Steel and Torrie, 1982).

Results from the RT assay were also observed to be heteroscedastic. The Box-Cox procedure (Box and Cox, 1964) indicated that a logarithmic transformation stabilized the variance ($\log_{10}$ [x+1]) was used to avoid taking the logarithm of zero. Means of transformed data were untransformed for presentation.

RESULTS and DISCUSSION

Two of the five Group II strains, USDA 142 and USDA 191, induced obvious RT symptoms beginning at 29 DAP (Table 1). Symptom onset and severity was more acute with USDA 191 than with USDA 142. By 50 DAP both treatments exhibited severe meristematic chlorosis and increased axillary shoot production. Additionally, plants nodulated by USDA 191 exhibited stem and petiole necrosis.

The total chlorophyll (a + b) content of the chlorotic treatments (USDA 142 and USDA 191) was significantly diminished relative to non-chlorotic treatments by 36 DAP (Table 1). This effect was more pronounced with USDA 191 than with USDA 142. At 50 DAP, all non-chlorotic Group II treatments maintained equal or, in the case of USDA 217, greater total chlorophyll per plant relative to the Group I/Ia strains. The concentration of total chlorophyll in the leaf tissue of plants nodulated with USDA 191 was statistically equal to that produced by USDA 110 through 43 DAP (Table1). When comparing within the non-chlorotic treatments, plants inoculated with USDA 110 generally had the lowest concentration of total chlorophyll except at the 22 day harvest (Table 1).

Nodulation with USDA 191 did not increase mean shoot dry weight relative to the noninoculated controls at the 22 harvest (Table 2). Although greater than the noninoculated control, dry weight. Accumulation with USDA 142 was significantly lower than the non-chlorotic treatments beginning at 43 DAP. With the exception of USDA 217, Group II strains produced significantly less biomass at 50 DAP than did USDA 206.

The total leaf protein (soluble + insoluble) content for the USDA 191 treatment remained equal to the noninoculated control, being statistically

lower than all other treatments beginning at 36 DAP (Table 2). The total protein content of plants nodulated by USDA 142 was similar to those of the non-chlorotic treatments until 50 DAP time at which a significant decrease was observed. Conversely, the concentration of soluble leaf protein for USDA 191 was significantly greater than all other treatments beginning 36 DAP (Table 2). Strain USDA 110 yielded soluble leaf protein concentrations that were consistently low relative to the other treatments after the 22- day harvest, although these differences were not statistically significant. Insoluble leaf protein concentrations in plants nodulated by USDA 191 were similar to those for the other inoculation treatments until 50 DAP.

Concentrations of soluble and insoluble protein for the noninoculated controls were generally higher and lower, respectively, than the inoculated treatments beginning at 36 DAP.

At the final harvest, leaflets of plants nodulated by USDA 142 were separated into two groups: chlorotic (≥ 10% chlorosis) and non-chlorotic (<10% chlorosis). The concentration of total chlorophyll in the chlorotic samples was nearly an order of magnitude less than that of the non-chlorotic leaflets (Table 3). Furthermore, the total protein concentration of the chlorotic leaflets was significantly less than that of the healthy tissue. This reduction in total protein was due primarily to a decrease in soluble leaf protein concentrations.

Strain USDA 110 consistently fixed more N_2 relative to all other treatments, although these differences were not always significant (Table 4). The kjeldah N content for USDA 191 was significantly higher than the noninoculated control by 43 DAP. However, the N content for the USDA 191 treatment at 50 DAP accounted for less than 35% of the total N_2 fixed by any of the other strains examined. The N content produced by USDA

Table 1. Plant chlorosis ratings and leaf chlorophyll of plants nodulated by *Bradyrhizobium japonicum* strains differing in rhizobitoxine phenotype at 29, 36 and 50 days after planting.

USDA Strain	Chlorosis rating [a]			Leaf Chlorophyll Content [b] (mg plant^{-1})			Leaf Chlorophyll Concentration [c] (mg 100 Cm^{-2})		
	29	36	50	29	36	50	29	36	50
38	0.0	0.0	0.0	0.68	4.34	16.15	1.23	3.04	3.33
110	0.0	0.0	0.0	0.89	4.93	16.07	1.01	2.40	3.11
138	1.1	3.1	5.1	0.77	3.40	5.32	1.00	2.73	1.58
142	3.1	5.1	5.1	0.38	0.97	0.62	0.86	2.05	0.77
191	0.0	0.0	0.0	0.91	5.31	19.02	1.17	2.93	3.16
206	0.0	0.0	0.0	0.64	4.85	16.01	0.57	2.10	2.58
217	0.0	0.0	0.0	0.79	5.51	16.34	1.00	3.09	3.13
NI[e]	0.0	0.0	0.0	0.27	0.54	ND[f]	0.68	1.10	ND[f]

(i) L.S.D = 0.64 (P = 0.05) (Statistical Analysis was limited to chlorosis data for USDA 142 and USDA 191)'
(ii) L.S.D = 1.07 (P = 0.05)' (iii) L.S.D = 0.33 (P = 0.05);
(e) NI = noninoculated controls; (f) ND = not determined.

Table 2. Shoot dry weight, leaf protein content and concentrations of soybeans nodulated by strains of *Bradyrhizobium japonicum* differing in rhizobitoxine phenotype at 22,36 and 50 days after planting.

USDA Strain	Shoot dry Weight [a] (g plant^{-1})			Total leaf protein content[b] (mg plant^{-1})			Soluble leaf protein concentra-tion[e] (mg g^{-1} fw leaf)		Insoluble leaf protein concentra-tion[d] (mg g^{-1} fw leaf)	
	22	36	50	22	36	50	36	50	36	50
38	0.34	0.80	2.72	46.8	156.0	744.1	50.5	54.8	33.2	59.4
110	0.34	1.02	2.77	46.7	183.9	789.4	44.5	55.0	27.0	59.5
138	0.32	0.85	1.75	48.6	151.9	521.6	51.2	53.8	33.2	61.4
142	0.29	0.43	0.55	43.2	76.2	169.2	71.8	71.0	31.6	67.4
191	0.35	1.10	3.01	48.1	179.2	867.9	48.4	49.3	28.7	60.0
206	0.33	1.11	3.32	48.0	203.4	876.8	42.7	49.1	25.0	57.5
217	0.33	0.96	2.99	44.8	179.1	790.6	47.3	56.1	30.7	59.6
NI[f]	0.35	0.53	ND[g]	41.3	70.6	ND	70.7	ND	20.8	ND

(i) L.S.D = 0.26 (P = 0.05) ; (ii) L.S.D = 35.9 (P = 0.05);
(iii) L.S.D = 6.7 (P = 0.05); (iv) L.S.D = 6.7 (P = 0.05);
(f) NI = noninoculated controls; (vii) ND = not determined.

Table 3. Selected comparisons of leaf tissue from soybean plants nodulated by *Bradyrhizobium japonicum* strain USDA 142 at 50 days after planting and grouped as to their degree of rhizobitoxine – induced chlorosis[a].

Parameter	Tissue classification[b] (mg g^{-1} fw leaf)	
	Chlorotic	Non-Chlorotic
	0.31a	2.27b
Total chlorophyll concentration	102.02a	132.42b
Total leaf protein concentration	43.81a	66.29b
Soluble leaf protein concentration	58.22a	66.14a
Insoluble leaf protein concentration		

(i) Means within a row with no letters in common are statistically different (p = 0.05).

(ii) Chlorotic tissue is classified as ≥ 10% chlorotic, non-chlorotic tissue is classified as < 10% chlorotic.

Table *4*. Total shoot kjeldahl nitrogen content and concentration in soybeans nodulated by *Bradyrhizobium japonicum* strains differing in rhizobitoxine phenotype at 36, 43 and 50 days after planting.

USDA Strain	Shoot Kjeldahl Nitrogen content [a] (mg N $plant^{-1}$)			Shoot Kjeldahl Nitrogen concentration[b] (% N)		
	36	43	50	36	43	50
38	30.98	51.94	88.49	3.96	3.29	3.29
110	39.06	58.05	86.95	3.96	2.82	3.15
138	38.42	57.61	77.72	4.56	4.35	4.51
142	13.51	19.74	55.05	3.23	3.37	4.12
191	44.14	63.95	112.83	4.02	3.43	3.65
206	47.55	73.95	116.56	4.31	3.50	3.54
217	41.59	64.58	98.38	4.39	3.42	3.30
NI[d]	4.99	5.21	ND[e]	0.97	0.98	ND

(i) L.S.D = 10.70 (P = 0.05) ;
(ii) L.S.D = 0.47 (P = 0.05);
(d) NI = noninoculated controls;
(e) ND = not determined.

142 was comparable to that for USDA 138 until 50 DAP. With the exception of USDA 217, Group II strains tended to fix less N_2 than Group I/Ia strains.

The concentration of kjeldahl N (Table 4) in the chlorotic treatments displayed two unique trends. The N concentrations for USDA 142 conformed to those for the non-chlorotic treatments until 36 DAP, at which time they deviated significantly and remained consistently greater than those for the remaining treatments. Secondly, although USDA 191 produced low N concentrations at 29 and 36 DAP, by 50 DAP the concentration was substantially higher than all non-chlorotic treatments.

Rhizobitoxine contents in nodules containing USDA 191 and 142 were greatest at 36 and 43 DAP, respectively (Table 5). These contents paralleled the appearance of severe symptoms mentioned above. It appears from this data that USDA 191 tended to accumulate greater quantities of nodular RT earlier in plant development than USDA 142. The RT concentration for USDA 191 nodules remained relatively constant between 22 and 50 DAP (Table 5). Without significance, a trend in the data suggesting a decrease in nodular RT concentration after the appearance of severe RT symptoms was observed. Detectable quantities of nodular RT were noted for several observations involving strains USDA 217 and 138, the levels of which ranged between 31 and 99 nmol per plant. However, no accompanying visual symptoms were observed, and the mean concentrations for these treatments were below the detectable limit. Despite a lack of chlorosis development, RT was detected at 22 DAP in nodules produced by USDA 142 and USDA 191.

Total nodules mass produced by strains USDA 191 and 142 at 50 DAP was significantly less than all other treatments (Table 6). However, non-chlorotic Group II treatments produced more nodule mass than the highly effective strain USDA 110. It is interesting to note that Group II strains consistently produced twice the number of nodules as the Group I/Ia strains at 50 DAP (Table 6). Nodule number also remained constant over time in Group I/Ia treatments whereas a gradual increase in number was observed in Group II treatments.

Leghemoglobin contents and concentrations (Table 7) of nodules containing USDA 191 were substantially lower than that of all other treatments beginning at 36 DAP. Relative to the non-chlorotic treatments, a significant decrease in the Lb content of USDA 142 nodules was observed at 50 DAP. Concentrations of Lb associated with USDA 142 were similar to those for USDA 38, 138, 110 and 217. The Lb concentrations produced by USDA 206 were significantly less than those for USDA 142 and all non-chlorotic treatments at 50 DAP.

Table 5. Total nodular rhizobitoxine (RT) content and concentration of soybean plants nodulated by *Bradyrhizobium japonicum* strains USDA 142 and USDA 191[a]

Days after planting (DAP)	RT content (n mol as n eqv plant^{-1})		RT concentration (n mol as n eqv g^{-1} fw nodule)	
	USDA 142	USDA 191	USDA 142	USDA 191
22	35 ab	270 bc	0.18X	1.35 yz
29	ND [b] [c]	389 C	ND[b][c]	1.31z
36	314C	674C	0.50 xyz	1.84z
43	749C	441C	0.70 xyz	0.58xyz
50	394C	307C	0.28xy	0.48xy z

(i) Means for a given variable with no letters in common are statistically different ($P = 0.05$) ;
(ii) Denotes one or more of the observations within a treatment yielding toxin content above the detectable limit.
(iii) ND = not detected (Although detectable RT levels were observed in one or more observations, the overall treatment mean was below the detectable limit).

Table 6. Nodule dry weight and number from soybeans nodulated by *Bradyrhizobium japonicum* strains differing in rhizobitoxine phenotype at 36,43 and 50 days after planting.

USDA Strain	Nodule dry weight [a] (mg plant^{-1})			Nodule number[b] (no. plant^{-1})		
	36	43	50	36	43	50
38	96.5	168.4	313.3	58.8	83.6	97.1
110	157.7	245.0	386.5	76.8	68.6	123.6
138	117.2	156.7	203.9	81.3	87.1	119.1
142	71.3	93.0	85.1	69.1	98.1	103.3
191	147.8	194.9	329.4	66.8	75.1	114.3
206	134.5	187.5	273.3	56.1	54.8	53.3
217	175.2	274.0	431.1	58.3	65.1	66.6

(i) L.S.D = 31.0 ($P = 0.05$) ;
(ii) L.S.D = 25.9 ($P = 0.05$);

The soluble nodular protein contents for USDA 142, 191 and 206 were substantially less than all other treatments at 50 DAP (Table 7). However, the concentrations of soluble protein in the chlorotic treatments generally paralleled those found in all other treatments with the exception of the USDA 206 treatment (Table 7). The protein concentration for USDA 206 nodules at 50 days was significantly less than all other treatments.

Table 7. Leghemoglobin (Lb) and soluble nodular protein content and concentration in soybean root nodules occupied by *Bradyrhizobium japonicum* strains differing in rhizobitoxine phenotype at 36,43 and 50 days after planting.

USDA Strain	Lb content[a] (mg Lb plant^{-1})			Lb concentration[b] (mg Lb g^{-1} fw nod)			Soluble nodular protein content[c] (mg protein plant^{-1})			Soluble nodular protein concentration[d] (mg protein g^{-1}fw nod)		
	36	43	50	36	43	50	36	43	50	36	43	50
38	1.77	2.24	5.69	2.73	1.94	2.72	11.80	16.96	33.57	18.16	14.15	16.07
110	2.31	2.35	5.42	2.60	1.68	2.18	13.98	19.94	39.69	15.80	13.86	15.92
138	1.71	2.25	3.30	2.65	2.08	2.26	11.73	15.30	26.89	18.18	14.10	18.54
142	0.76	0.70	0.73	2.04	0.90	0.97	9.25	10.66	11.00	25.05	13.98	16.87
191	1.97	3.52	5.36	2.44	2.48	2.57	12.55	18.82	33.04	15.60	13.25	15.90
206	2.70	3.93	5.46	3.40	3.55	3.41	14.18	18.43	36.63	17.90	16.97	22.82
217	2.47	3.17	3.21	2.87	2.41	1.56	12.37	16.83	24.11	14.33	12.93	11.69

(i) L.S.D = 0.67 (P = 0.05) ;
(ii) L.S.D = 0.45 (P = 0.05);
(iii) L.S.D = 2.31 (P = 0.05);
(iv) L.S.D = 2.58 (P = 0.05);

Relative to the non-chlorotic treatments, significant declines in total nitrogenase activities were observed for plants nodulated by USDA 191 and USDA 142 at 36 and 50 DAP, respectively.

DISCUSSION

Although the causative mechanism requires further study, it is clear that nodulation by chlorosis-inducing Group II strains drastically reduced the accumulation of both chlorophyll a and b in leaf tissue. This reduction was accompanied by a decline in general shoot productivity as estimated by shoot dry weight, leaf surface area, and leaf protein production.

High levels of toxin production and chlorosis development were delayed in plants nodulated by USDA 142 relative to those nodulated by USDA 191. As a result, USDA 142 yielded greater chlorophyll, leaf surface area and shoot dry weight estimates at 36 DAP when compared to USDA

191. In addition, when leaf tissues of plants nodulated by USDA 142 were segregated into chlorotic and non-chlorotic groups, the latter accounting for 44% of the total leaf fresh weight, leaf soluble protein and chlorophyll concentrations in the non-chlorotic tissue were significantly greater than those of the chlorotic tissues. It is conceivable that the resulting increase in photosynthetic capacity resulting from nodulation by USDA 142 relative to USDA 191 may assist the former plants in overcoming RT-induced symptoms (El-Sayed, 1995 & 1998).

The reductions in leaf protein of the chlorotic treatments are reflected in the reductions in shoot kjeldahl N measurements. Fuhrmann (1990) reported significantly higher concentrations of shoot nitrogen in plants where RT symptoms were expressed. This same trend is observed in the leaf protein data of plants nodulated by USDA 191 but not for USDA 142. It is possible that this discrepancy may reflect the greater photosynthetic capacity for the USDA 142. treatment resulting from the delay in symptom expression described above. Increased carbon fixation would conceivably lead to larger plants decreasing the concentration of protein in the tissue. The increase in kjeldahl nitrogen concentrations of the severely chlorotic treatments relative to the non-chlorotic treatments also suggests that N_2 fixation was occurring at a low level with little or no increase in biomass (Abdel-Mawly and El-Sayed, 1999).

Carbon fixation in soybean is facilitated by the enzyme ribulose 1,5-bisphosphate carboxylase / oxygenase (rubisco), an enzyme which can constitute as much as 50% of leaf soluble protein (Wittenbach *et al.*, 1980; El-Sayed and Abdel-Mawly, 1999). Furthermore, the regeneration of ribulosebisphosphate depends on the supply of ATP and $NADPH_2$ from photosynthesis (Farquhar *et al.*, 1980; Von Caemmerer and Farquhar, 1981). Plant growth is therefore dependent on the effectiveness of the entire photosynthetic system. Considering the low concentrations of soluble protein in the chlorotic tissues for USDA 142, a logical course would be to assess the effect of RT on the production and function of rubisco itself. Such information may help define the mechanistic role of RT as a growth limiting compound.

The results of this study clearly suggest that high levels of nodular RT are required to induce severe foliar chlorosis in Giza 82 soybean shoots. This conclusion is supported by the elevated levels of the toxin in the nodule coinciding with the appearance of severe visual symptoms and the lack of symptoms for treatments producing lower amounts of RT. Nodules produced by USDA 191 contained an average of 271 n mol RT plant^{-1} at 22 DAP without inducing noticeable chlorosis. It is likely that the transport of a threshold amount of toxin necessary for chlorosis induction had not yet reached the meristematic regions of the shoot at this early stage in plant and nodule development.

Physical characteristics of nodule senescence include browning of the nodule surface followed by the eventual disintegration of the nodular tissues (Sutton, 1983; Abdel-Aziz *et al.*, 2003). Often observed at these advanced stages are the empty hulls of the nodule cortex attached to the roots (Allen and Allen, 1958). Biochemical changes in the nodule during senescence include reductions in nitrogenase activity (Paau and Cowles, 1979), leghemoglobin content (Virtanen *et al.*, 1947), soluble nodular protein levels (Nash and Schulman, 1976) and increases in proteolytic activity in the nodule cytosol (Pfeiffer *et al.*, 1983; El-Sayed and Abo-El-Wafa, 2001).

Studies by Lawn and Brun (1974) and kollman *et al.* (1974) have led to the suggestion that carbohydrate deprivation, or the ratio of carbohydrates to nitrogen supply, may control the onset of senescence (Sutton, 1983). In more recent studies, interruption of the phloem supply by way of defoliation, nodule detachment, or stem girdling resulted in O_2 limited declines in nitrogenase activity (Hartwing *et al.*, 1987; Hunt *et al.*, 1987; Vessey *et al.*, 1988; Mohamed and El-Sayed, 2003). Walsh *et al.*, (1989) have shown that a reduction of water flow to the nodule, via disruption of the phloem, reduced both nitrogenase activity and xylem export of nitrogenous compounds from the nodule (Mc Clure and Israel, 1979).

The accelerating effect of RT-induced chlorosis on nodule senescence has apparently not been reported in the literature. During the present study, many nodules of USDA 191 were found to be brown and necrotic starting at 36 DAP. No such symptoms were observed in any other treatment. The appearance of necrosis in the nodules of USDA 191 corresponded with reductions in total nitrogen activity, concentrations of Lb

and soluble nodular protein, followed by a reduction in nodule mass. Similar patterns were not observed with USDA 142, presumably as a result of delayed symptom expression in the shoot and subsequent increase in photosynthetic capacity. Although declines in soluble nodular protein and Lb for the USDA 206 treatment may suggest the onset of nodule senescence, other symptoms typical of senescence were not evident. Fuhrmann and Wollum (1989) previously reported that USDA 206 produces relatively high nodule weights. Considering the relatively large dry weights of USDA 206 nodules, the reduced protein and Lb concentrations in these nodules are more likely a reflection of a dilution effect due to the production of polysaccharides or other low N substances in the nodule.

CONCLUSIONS

With the exception of USDA 38, RT was detected in the nodules of all group II strains tested. However, only nodules containing strains USDA 142 and USDA 191 produced quantifiable amounts of RT and, consequently, were the only two treatments displaying RT-induced chlorosis. Group II strains tended to fix less total N than Group I/Ia strains, although similar trends are not reflected in the accumulation of nodule proteins. Group II strains consistently produced a greater number of nodules than did Group I/Ia. Coinciding with the appearance of moderate to severe symptoms,there were reductions in total leaf chlorophyll, leaf protein, shoot and nodule mass, and total N contents of the affected plants. During extended periods of severe chlorosis, reductions in Lb and soluble nodular protein were observed. It appears that, in the presence of severe RT- induced chlorosis, reductions in total plant productivity occur mainly as a result of decreased photosynthetic capacity of the plant and, subsequently, a reduction in N_2 fixation. The data presented here suggest that, in the absence of discernable visual symptoms, the impact of nodulation by RT^+ (Group II) strains on short term soybean productivity is minimal

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الملخص العربي

تأثير Rhizobitoxine على صفات النمو والمحصول وبعض الخواص البيوكيميائية في فول الصويا

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تختلف سلالات بكتيريا *Bradyrhizobium Japonicum* في إنتاج مادة Rhizobitoxine (RT^+) بكميات مختلفة كما تختلف فى قدرتها على حدوث اصفرار نتيجة لنقص الحديد في نباتات فول الصويا صنف جيزة 82. فقد تم تقييم إنتاجية كل من السيقان، والعقد البكتيرية، وتثبيت النيتروجين ونسب البروتين بالعقد الجذرية تحت ظروف الصوبة الزجاجية خلال فترة النمو. لقحت بذور فول الصويا بمعلق من المجموعة الثانية من سلالات USDA وهى 38، 138، 142، 191، 110، 206، 217، سلالات USDA 110، 206 تشتمل على الكنترول. زرعت النباتات فى غياب النيتروجين لمدة خمسون يوماً فى بيئة من مادة الفيرميكوليت، وبعد 22 يوماً من الزراعة تم التقييم أسبوعيا لكل من الكلوروفيل ، ونسب البروتين بالأوراق، وكتلة الخلايا الحية، ومحتوى العقد من الهيموجلوبين، والبروتين الذائب ، RT ، والمحتوى الكلى للنيتروجين في السيقان .

تم الكشف عن Rhizobitoxine فى العقد للسلالات المنتجة لـ RT^+ باستثناء سلالة USDA 38، ومع ذلك فإن كل من سلالتي USDA 124، 191 تنتج فقط تركيزات قليلة من RT ممكن قياسها.

أوضحت النتائج حدوث اصفرار متوسط متوافق مع نقص في تركيز الكلوروفيل لكل من السيقان، والوزن الجاف للعقد البكتيرية والتثبيت الكلي للنيتروجين. أثناء حدوث الاصفرار لفترات طويلة يحدث انخفاض للهيموجلوبين ونسبة البروتين الذائب بالعقد البكتيرية ، ونتيجة لتجمع الكربون فإن جميع المعاملات التي لم يحدث بها اصفرار حللت إحصائياً وأعطت إنتاجاً معنوياً مرتفعاً عن المعاملات التي حدث بها اصفرار. وبالمثل فإن معاملات المجموعة الثانية الغير مصفرة تقوم بتثبيت كمية قليلة من الكربون بالنسبة إلى الكنترول ، وبالرغم من ذلك فإن هذه الاختلافات غير معنوية إحصائياً.