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Some Phenolic Compounds Enhance Nodulation and Nitrogen Fixation in a Soybean / *Bradyrhizobium japonicum* System

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Summary

ABD-ALLA M. H. 1994. Some phenolic compounds enhance nodulation and nitrogen fixation in a soybean / *Bradyrhizobium japonicum* system. – Phyton (Horn, Austria) 33: (2): 249–256. – English with German summary.

The present study has been carried out to clarify the effect of type, concentration and method of application of phenolic compounds (p-hydroxybenzoic acid, o-dihydroxybenzene, and 1,3,5-trihydroxybenzene) on nodulation, plant growth and total plant nitrogen. Phenolic compounds levels of 10,100 and 1000 µM were applied in two different modes; spraying of the shoots system and application with the irrigation solution. This study revealed that the three classes of phenols (mono-, di-and tri-) each at concentrations of 100 µM significantly increased nodulation, plant dry matter and total plant nitrogen when applied with the irrigation solution. At this concentration, 1,3,5-trihydroxybenzene significantly increased nodule NADH-GOGAT, NADH-GDH activities and total soluble protein. However, mono- and di-phenols caused only a slight increase in the total activities of these enzymes and in protein. The promotive effect of phenolic compounds at lower concentrations, on nodule formation, plant growth and enzymes of ammonia-assimilation are promising for using these substances to enhance the nitrogen status of soybean.

Zusammenfassung

ABD-ALLA M. H. 1994. Einige phenolische Substanzen fördern die Knöllchenbildung und Stickstoffixierung in einem Sojabohen- / *Bradyrhizobium japonicum*-System. – Phyton (Horn, Austria) 33 (2): 249–256. – Englisch mit deutscher Zusammenfassung.

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Gegenstand vorliegender Arbeit war, den Einfluß phenolischer Substanzen (p-Hydroxibenzoesäure, o-Dihydroxibenzen, und 1,3,5-Trihydroxibenzen) bei verschiedenen Konzentrationen und Applikationsmethoden auf Knöllchenbildung, Wachstum der Pflanzen und dem Gesamtstickstoffgehalt abzuklären. Phenolische Verbindungen wurden in Mengen von 10, 100 und 1000 µmol auf zweierlei Weise appliziert; durch Besprühen des Sproßsystems bzw. mittels der Gießlösung. Es zeigte sich, daß jede der drei Phenolklassen (mono-, di- und tri-) bei der Konzentration von 100 µmol die Knöllchenbildung das Trockengewicht und den Gesamtstickstoff der Pflanzen signifikant erhöht. Soferne die Substanzen mit der Gießlösung appliziert wurden. Bei dieser Konzentration steigerte 1,3,5-Trihydroxibenzen signifikant die Aktivitäten der NADH-GOGAT und NADH-GDH von Knöllchen, aber auch das gesamte lösliche Protein. Mono- und Diphenole bewirkten jedoch bloß einen leichten Anstieg der Gesamtaktivitäten dieser Enzyme und des Proteins. Der fördernde Einfluß der phenolischen Komponenten bei geringer Konzentration auf Knöllchenbildung, Pflanzenwachstum und Enzyme der Ammoniumassimilation erscheinen verheißungsvoll für deren Anwendung, um Stickstoffverbindungen in Sojabohnenpflanzen zu steigern.

Introduction

Phenolic compounds are important in the regulation of plant growth and metabolism (JAIN & SRIVASTAVA 1984, NANDAKUMAR & RANGASWAMY 1985). Phenolic compounds can either be synergistic or antagnoistic towards auxin on rooting (PAL & NANDA 1981). They induce growth of daughter fronds of *Lemna* (DEKOCK & al 1974), bud formation in tobacco cells (LEE & SKOOG 1965), flowering in *Lemna* and *Zea* (CLELAND & TANAKA 1979, ASTHANA & SRIVASTAVA 1978) and, at lower concentrations, may increase nitrogen content, nitrate reductase and glutamate dehydrogenase activities,but may inhibit these parameters at higher concentrations (JAIN & SRIVASTAVA 1981 a,b,c). Reports on the effect of phenolic compounds on nodulation and N2-fixation of some legumes are, however scarce (DHIR & RAO 1989). We have studied the effect of type, concentration and method of application of phenolic compounds on nodulation and plant growth of soybean. Glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) activities were also assessed under the influence of phenolic compounds.

Materials and Methods

Plant culture and experimental conditions:

Surface-sterilized soybean seeds (*Glycine max* L. cv. Clark) were inoculated with 5 ml of *Bradyrhizobium japonicum* RCR 3407 suspension (approximately 10^7 cells/ml) per five seeds and were planted into plastic pots (17 cm diameter) contaning 3 kg autoclaved clay soil. Some physical and chemical characteristics of the soil were described previously (ABD-ALLA 1992). Seedlings were thinned to two per pot after five days. Plants were grown in a wire proof greenhouse. Temperatures during the experiment averaged 36 / 29 °C (day/night) and the relative humidity ranged from 50 to 80 %.

Three classes of phenolic compounds, i.e. monophenol (p-hydroxybenzoic acid), diphenol (o-dihydroxybenzene) and polyphenol (1, 3, 5-trihydroxybenzene) were tested at concentrations of 10, 100 and 1000 µM. Two different methods of application of the compounds have been used in the present study, i.e. spraying of the shoot system and application with the irrigation solution. For application by spraying method, 20 ml of the phenolic solution were applied to the shoot system of the growing plants in each pot. Immediately before application a few drops of a surfactant (Tween'80) were added to the aqueous solution. Plants were sprayed every 3 d for 4 weeks after planting. For application of the irrigation methods, phenolic solutions were given twice a week with the irrigation solution during 4 weeks after planting. All pots were flushed daily with $^{1}/_{4}$ strength nitrogen- free Hoagland solution. Plants not receiving phenolic solutions were used as a control. Treatments were arranged in a randomized block design with three replicates each. Plants were harvested 66 days after planting. At this time samples were large enough for the analysis of nodules. Nodule number, nodule fresh weight, shoot and root dry weights were recorded. Nitrogen content of the plant tissues were determined by the Kjeldahl method (BLACK & at 1965).

Preparation of cell-free extracts of nodules:

Samplings of excised nodules (500 mg) were extracted by hand at 0 to 3°C in a ground glass homogenizer with 5 ml extraction buffer containing 150 µM phosphate buffer (pH 6.9), 1.2 mM sucrose, 6 µM EDTA and 3 µM cysteine. The resulting homogenates were centrifuged at 3.500 × g for 8 min, to remove nodule debris. The supernatant was centrifuged at 12,000 × g for 20 min. The supernatant of the second centrifigation was assayed for plant enzyme activities and soluble protein.

Enzyme assays:

The NADH-dependent glutamate dehydrogenase (NADH-GDH) and glutamate synthase (NADH - GOGAT) activities were assayed spectrophotometrically following the oxidation of NADH (BULLEN 1956).The assay mixture consisted of 40 mM phosphate buffer (pH 8). Substrate concentrations for NADH - GDH assays were 100 µM NADH, 2.5 mM α-ketoglutarate, and 200 mM $(NH4)_2$ SO4. NADH - GOGAT activity was measured using 100 µM NADH,2.5 mM α-ketoglutarate and 10 mM L-glutamine. The reaction was started by adding enzyme preparation, and the decrease in absorbance was recorded continuously for 5 min at 340 nm in a double beam spectrophotometer (Spectronic 2000, BAUSCH & LOOMB).

Protein determination:

Soluble protein was measured according to LOWRY & al 1951.

Statistical analysis:

Differences in nodule number, nodule fresh weight, dry mass nitrogen contents of plant tissues, GDH, GOGAT and soluble protein contents of nodules were tested for statistical significance between types and concentrations of application of phenolic compounds, using one-way analysis of variance of means (PC-State computer program).

Results and Discussion

Nodulation and plant growth:

Data presented in Table 1 and 2 show that nodule number increased significantly on plants treated with 100 μM of the three phenolic compounds tested. Polyphenol was found to be most effective in nodule formation. The average number of nodules formed in the presence of p-hydroxybenzoic acid was less than the number formed in the presence of 1,3,5-trihydroxybenzene. The increase in nodule number was accompanied by an increase in fresh mass. The results obtained for dry weight of roots and shoots showed similar trends. Calculation of nodule number per gram dry weight root generally shows that the treatment rather had an effect on nodule formation than on growth. Calculation of shoot/root ratio on a dry weight basis shows that the dry matter increment as a result of the treatment is equally partitioned between shoot and root. Likewise, the nitrogen content per plant increased with application of all phenolics when applied at 100 μM. Application of phenolic compounds at 100 μM with the

Table 1

Effect of phenolic compounds applied with the irrigation solution on nodulation and plant growth of soybean*

Phenolic compounds μM	Nodules/plant		nodule no. /g root	Dry weight (g/plant)		Shoot /Root ratio	Total nitrogen (mg/plant)	
	Number	Fresh wt (g)		Shoots	Roots		Shoots	Roots
Control	30.7 d	1.10 b	21.6	7.9 e	1.42 c	5.6	283 b	60b
p-Hydroxybenzoic acid								
1000	21.7 e	0.99 b	19.6	6.0 f	1.07 d	5.6	214 c	45 d
100	46.0 b	2.04 a	28.4	9.1 cd	1.62 b	5.6	310 b	68 b
10	29.0 d	1.05 b	20.6	8.0 e	1.41 c	5.6	286 b	59 c
o-Dihydroxybenzene								
1000	20.7 e	0.96 c	18.9	6.1 f	1.09 d	5.6	219 c	46 d
100	61.0 a	2.28 a	32.9	10.4 b	1.85 b	5.6	371 a	77 a
10	29.6 d	1.24 b	20.0	8.3 de	1.48 c	5.6	295 b	62 b
1, 3, 5-Trihydorxybenzene								
1000	19.7 e	0.75 c	20.7	5.3 g	0.95 d	5.6	189 c	40 d
100	66.0 a	2.20 a	31.3	11.5 a	2.11 a	5.5	411 a	88 a
10	38.3 c	1.31 b	18.2	8.6 de	1.93 a	4.1	308 b	71 a

* Each values represents the mean of three replicates. Values in the same column followed by the same letter are not significantly different at the 5% level by DUNCAN'S multiple range test.

irrigation solution (Table 1) was more effective than application by the spraying method (Table 2).

Apparently, all three classes of phenols (mono-,di-,and tri-) enhanced nodulation, plant growth and nitrogen status of soybean. These results are in agreement with the results of DHIR & RAO 1989 who reported that foliar spraying of phenolic compounds increased nodulation, plant growth and nitrogen yield of pigeonpea. SINGH & al. 1978, 1986 observed that phenolic compounds increase flower, pod and grain number per plant. WAIN & TALYOR 1965 reported that the presence of two OH groups at the ortho-position was necessary for the growth promotory activity of phenols. The present data show that nodulation is enhanced even by monophenols which contain only one hydroxyl group.

Higher concentrations of the phenolic compounds tested (1000 μM) significantly reduced nodule number, nodule fresh weight, plant growth and nitrogen yield of soybean (Tables 1 and 2). The inhibition of nodulation and growth by high concentrations of phenols could be attributed to their

Table 2

Effect of phenolic compounds applied by spraying on modulation and plant growth of soybean.*

Phenolic Compounds μM	Nodules/plant		nodule no. /g root	Dry weight (g/plant)		Shoot /Root ratio	Total nitrogen (mg/plant)	
	Number	Fresh wt (g)		Shoots	Roots		Shoots	Roots
Control	30.7 b	1.10 c	21.6	7.9 c	1.42 bc	5.6	283 c	60 b
p-Hydroxybenzoic acid								
1000	25.7 c	1.07 c	21.8	6.6 d	1.18 d	5.6	236 de	47 c
100	39.6 b	1.87 b	26.1	8.5 c	1.52 b	5.6	304 b	60 b
10	29.0 c	1.00 c	20.4	7.9 c	1.42 bc	5.6	282 b	58 b
o-Dihydroxybenzene								
1000	29.3 b	0.98 c	21.7	7.6 c	1.35 c	5.6	271 d	41 c
100	50.3 a	2.07 a	30.7	9.2 b	1.64 b	5.6	329 b	63 a
10	28.7 c	1.08 c	20.2	7.9 c	1.42 bc	5.6	282 c	60 b
1, 3, 5-Trihydroxybenzene								
1000	23.1 c	0.86 d	20.4	6.3 d	1.13 d	5.6	225 c	42 c
100	51.7 a	2.04 a	27.8	10.3 a	1.86 a	5.6	368 a	68 a
10	37.7 b	1.10 c	25.8	8.2 c	1.46 bc	5.6	293 b	62 a

* Each values represents the mean of three replicates. Values in the same column followed by the same letter are not significantly different at the 5% level by DUNCAN'S multiple range test.

inhibitory effects on the over-all plant metabolism by non-specific inhibition of ATP synthesis (KHAN 1968) or membrane permeability (POSPISIL & al 1987).

Nodule glutamate synthase, glutamate dehydrogenase and soluble protein:

Ammonia is the primary product of biological N_2-fixation. In the nodules the assimilation of ammonia into amino acids occurs primarily via glutamine synthetase and glutamate synthase (OHYAMA & KUMAZAWA 1980). However, when in excess ammonia may be assimilated by glutamate dehydrogenase (STRIPF & WERNER 1978), low concentrations (100 μM) of 1,3,5-trihydroxybenzene caused a significant increase in the activities of NADH-GOGAT and NADH-GDH and in nodule soluble protein (Table 3). Similar results were obtained by ROTH-BEJERANO & LIPS 1970 and JAIN & SRIVASTAVA 1981 b, c. These authors reported that some phenolic compounds enhanced nitrate reductase and glutamate dehydrogenase activities. At 100 μM concentrations p-hydroxybenzoic acid and

Table 3

Effect of phenolic compounds applied with the irrigation solution on nodule NADH-glutamate dehydrogenase (NADH-GDH), NADH-glutamate synthase (NADH-GOGAT) activities and soluble protein contens in soybean. Values means of four replicates*.

Phenolic compounds μM	Enzyme activity NADH-GOGAT (μ mol NADH oxidized/min/g fw nodule)	NADH-GDH	Protein content (mg/g fw nodules)
Control	19.3 c	25.9 b	9.00 b
p-Hydroxybenzoic acid			
1000	13.6 d	18.3 c	8.80 b
100	20.3 bc	26.3 b	10.1 b
o-Dihydroxybenzene			
1000	14.3 d	17.7 c	9.20 b
100	20.9 bc	27.1 b	10.9 b
1, 3, 5-Trihydroxybenzene			
1000	12.3 d	18.9 c	8.90 b
100	27.8 a	31.8 a	14.2 a

* Means within columns followed by the same letter are not significantly different at the 5% probability level according to DUNCAN'S range test.

o-dihydroxybenzene did not cause a significant change in total enzyme activities and soluble protein. As these compounds enhance total plant nitrogen, it appears that NADH-GOGAT and NADH-GDH activities in the nodules are not "rate-limiting" for ammonia assimilation in the nodules under the experimental conditions of this study. However, higher concentration of phenolic compounds (1000 μM) had inhibitory effect on the enzyme activities greater than on nodule soluble protein (Table 3).

The results of this study indicate that phenolic compounds at low concentration (100 μM) can be used to improve crop performance by stimulating nodulation,plant growth and ammonia-assimilation. They suggest that application of phenolic compounds (polyphenols) at low concentration with the irrigation solution may have the potential to enhance the nitrogen status of soybean.

References

ABD-ALLA M. H. 1992. *Brabyrhizobium* strains and the nodulation , nodule efficiency and growth of soybean *(Glycine max L.)* in Egyptian soils. – W. J.Microbiol. Biotechnol.8: 593–597.

ASTHANA J. S. & SRIVASTAVA H. S. 1978. Effect of presowing treatment of maize seeds with salicylic acid and ascorbic acid on seedling growth and nitrogen content. — Indian J. Plant Physiol. 21: 150–155.

BLACK C. A., EVANS D. D. & EMSMINGER L. E. 1965. Methods of soil analysis. – Agron. 9, P.1171-1174 Amer. Soc.Agron., Inc. Madison WS.

BULLEN W. A. 1956. The isolation and characterization of glutamic dehydrogenase from corn leaves. – Arch. Biochem. Biophys. 62: 173–183.

CLELAND C. F. & TANAKA O. 1979. Effect of day length on the ability of salicylic acid to induce flowering in the long day plant *Lemna gibba* G3 and the short day plant *Lemna paucicostata* 6746. – Plant Physiol. 649: 421–424.

DEKOCK P. C., FLORA B. G. & INNES A. M. 1975. The effect of salicylic acid on the growth of *Lemna gibba*. – Annal. Bot. 38: 903-908.

DHIR K. K. & RAO L. 1989. The influence of some phenolic compounds on nodulation in pigeonpea (*Cajanus cajan* (L) Millsp). – Biol. Plant. 31: 44–50.

JAIN A. & SRIVASTAVA H. S. 1981a. Effects of salicylic acid on growth, nitrogen content and peroxidase activity in maize seedlings. – National Academy of Science (India) 51B: 311–317.

— & — 1981b. Effect of Salicylic acid on nitrate reductase activity in maize seedlings. – Physiol. Plant. 51: 339–342.

— & — 1981c. Effect of salicylic on nitrite reductase and glutamate dehydrogenase activities in maize roots. – Physiol. Plant. 53: 285–288.

— & — 1984. Effect of phenolic acid on anthocyanin content in maiz roots. – Biol. Plant. 26: 241–245.

KHAN A. A. 1968. Inhibition of gibberellic acid induced germination by abscisic acid and reversal by cytokinins. – Plant Physiol. 34:, 1461–1462.

LEE T. T. & SKOOG F. 1965. Effect of substituted phenols on bud formation and growth of tabacco tissue cultures. – Physiol. Plant. 18: 386–402.

LOWRY O. H., Rosebrough N. J., FARR A. L. & RANDALL R. J. 1951. Protein measurement with Folin phenol reagent. – J.Biol. Chem. 193: 265–275.

NANDAKUMAR L. & RANGASWAMY N. S. 1985. Effect of some flavonoids and phenolic acids on seed germination and rooting. – J.exp. Bot. 36: 1313–1319.

OHYAMA T. & KUMAZAWA K. 1980. Nitrogen assimilation in soybean nodules. I. The role of GS/GOGAT system in the assimilation of ammonia produced by N2 fixation. – Soil Sci. Plant. Nit. 26: 109–115.

PAL M. & NANDA K. K. 1981. Rooting of etiolated stem segments of *Populus robusta* - interaction of temperature, catechol and sucrose in the presence of IAA. – Physiol. Plant. 53: 540–542.

POSPISIL F., HRUBCOVA M. & CVIKROVA M. 1987. Anodic oxidation of coumaric and caffeic acid and their effects on nitrate uptake and nitrate reductase in *Nicotiana tobacum* cell suspension. – Biol. Plant. 29 : 175–192.

ROTH-BEJERANO N. & LIPS H. 1970. Hormonal regulation of nitrate reductase activity in leaves. – New Phytol. 69: 165–169.

SINGH P., GREWAL M. K. & MALIK C. P. 1986. Phenolic effects on reproductive development of moong (*Vigna radiata* (L) Wilczek). – J. Plant Sci. Research 2: 15–19. 19.

SINGH G., SEKHON N. & KAUR M. 1978. Effect of phenolic compounds on the yield of potential of gram *(Cicer arietinum L.)*. – Indian J. Plant Physiol. 23: 21–25.

STRIPF R. & WERNER D. 1978. Differentiation of *Rhizobium japonicum*. II. Enzymatic activities in bacteroids and plant cytoplasm during the development of nodules of *Glycine max*. – Z. Naturforsch. 33: 373–381.

WAIN R. L. & TALYOR H. F. 1965. Phenols as plant growth regulators. – Nature 207: 167–169.