Nodulation, Nitrogen Fixation and Growth of Soybean Plants (*Glycine max* Merr.) in Soil Supplemented with Chitin or Chitosan*

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Abstract : Pot soil was supplemented with varying concentration of chitin or chitosan to investigate their influence on the nodulation, nitrogen fixation and growth of soybean plants. Significant ($P=0.05$) reductions in nodule fresh weight and nitrogen fixation (measured as acetylene-reduction activity) were observed at the early growth stage (28 DAP) for plants grown in soil supplemented with chitin or chitosan as compared to regular pot soil. However, the nodule numbers were not affected at the early growth stage with the exception of 1% of chitin or chitosan. Both nodulation and nitrogen fixation activity at a later growth stage (42 DAP) were markedly enhanced in soils supplemented with 0.10% of either chitin or chitosan. These results suggest that all concentrations of chitin or chitosan have a severe influence on nodule development and N_2 -fixation activity at the early growth stage in soybean plants. The production of total dry matter was higher in untreated control plants at 28 DAP, while plants treated with 0.10~0.25% chitin or chitosan produced higher dry matter at 42 DAP. Soybean seed yield increased with the increasing pod number at these levels of treatment. However, the above parameters did not differ greatly between chitin and chitosan at corresponding levels of concentration.

Key words : Chitin, Chitosan, Nitrogen fixation, Nodulation, Soybean.

キチンまたはキトサンの土壌混和がダイズの根粒形成、窒素固定および生育に及ぼす影響: Muhammad ALI・堀内孝次・宮川修一 (岐阜大学大学院連合農学研究科)

要 旨 : 異なる濃度のキチンまたはキトサンを土壌に混和してダイズをポット栽培し、根粒形成、窒素固定ならびに生育への影響を調査した。両物質とも混和土壌では非混和土壌に比べて生育初期 (播種後 28 日) には根粒の生体重とアセチレン還元法で測定した窒素固定能の有意な低下が認められた。一方、根粒着生数は 1%濃度の処理では減少したが、0.10~0.50%濃度処理では影響が見られなかった。播種後 42 日にはキチンおよびキトサン両者ともに 0.10%混和土壌で根粒形成と窒素固定能の増加が見られた。以上の結果から、生育初期ではキチンとキトサンはどの濃度でも根粒形成と窒素固定に対して抑制的に作用することが示された。全乾物重は播種後 28 日目では、非混和土壌が最も高い値であったが、42 日目では 0.10 および 0.25%濃度混和の土壌で最も高かった。完全粒重は莢数の増加を伴ってそれらの濃度で高い値を示した。以上の効果についてキチンとキトサンの間には差異が認められなかった。

キーワード : キチン, キトサン, 根粒形成, ダイズ, 窒素固定.

Cultivation of leguminous crops in agricultural soil is a customary practice to improve soil fertility. The current practice for legume culture includes inoculation of seeds with rhizobia and treatment of seeds with fungicides to reduce seed rot and damping-off caused by soil-borne fungi. Application of fungicides to control diseases has been found to be toxic to rhizobial growth^{5,14)}, which hinder nodulation and reduce nitrogen fixation^{7,15,23)}. Use of chemical fungicides is not only detrimental to rhizobial bacteria but also cause the pollution of the environment, which is now a great global concern.

Soil amendment with organic matters has long been advanced as a biological method of disease control by plant pathologists. In recent years, farmers use ground shells of crab or lobster (as a source of chitin or chitosan) for soil amendment to control soil-borne diseases biologically. Nawakawa²²⁾ reported that about 3,000 t of ground crab or lobster shells are in use each year as organic fertilizer, or soil amendment in Japan. The addition of chitin to the soil increases rhizobial multiplication and nitrogen fixation⁴⁾. On the other hand, Polyanskaya²⁶⁾ contradicted this due to the antagonistic effects of actinocyetes. The above reports implicated the changes in the rhizobial population due to the addition of chitin in soil. However, it is well-established that the

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microbial complex changes with the amendment of soil by addition of chitin have been described elsewhere^{6,21,25}). As free living organisms, rhizobia interact with indigenous soil micro organisms, which may in turn influence the ability of rhizobia to become established in the soil^{2,12}). The failure of legume establishment due to unsuccessful nodulation has been attributed, at least in part, to antagonistic soil micro organisms^{13,30}). Although a lot of research work has been done on the biological control of soil-borne diseases and microbial processes associated with aminopolysaccharide (chitin or chitosan) amendment soil, no information is available on the nodulation and nitrogen fixation change in microbial equilibrium resulting from incorporation of aminopolysaccharide to soils. The objective of this investigation was to study the influence of chitin and chitosan incorporation to soil on the root nodulation, nitrogen fixation and growth of soybean plants.

Materials and Methods

The soil used in this experiment was the sandy loam (Gifu, Nagara river soils). The soil was strained by power-driven sieve to remove stones and other dust materials and air dried before amendment with chitin or chitosan. About 1,300g of air-dried soil (total C content 0.06%, total N content 0.16%, total P content 0.09% and pH 6.39) was amended by thorough mixing with 0.10, 0.25, 0.50, or 1% (w/w) chitin and chitosan (Katakura Chikkarin Company, Ltd.) separately. Nonamended soil was considered as control treatment (C). The soils were fertilized with 0.15g P_2O_5 and 0.16g K_2O pot^{-1} during amendment. The soil pH (soil : water = 1 : 2.5) was monitored at 2, 4, and 6 weeks after addition of chitin or chitosan. The pH of soil at 6 weeks was altered from 6.39 to 6.76, and 6.39 to 6.68 by the addition of 1% or 0.50% of chitin or chitosan, respectively. However, amendment with 0.25 ~ 0.10% resulted in almost no changes of soil pH in comparison with nonamended soil.

Plastic pots (1/10,000 a) with one drainage hole at the bottom were filled separately with amended and nonamended soil. Soybean seeds (cv. Akishrome) were sorted out on the basis of size and mass (0.30 ± 0.005 g per seed) before sowing, and were sown with four seeds per pot. The pot soils were not inoculat-

ed artificially by any rhizobial strains. The pots were arranged with a completely randomized design on benches that were kept in outdoor open places. The plants received ad libitum tap water as irrigation until the end of the experiment. Soybean seedling was thinned out to one per pot at 12 days after planting (DAP).

Plants were sampled at 28 and 42 DAP. At each sampling date, plants were excised at the ground level. The underground portions on plant roots were washed carefully to remove soil particles and plant debris, and then enclosed in a polyvinyl bag for one hour for measurement of nitrogen fixation by acetylene reduction method³¹). After the measurement of acetylene reduction activity (ARA) of root nodules, nodules were carefully separated from roots, washed with water and blotted by filter paper to remove water. Then number and fresh weight were recorded. Roots, together with nodules, and plant tops (leaves & stems) at 28 and 42 DAP were oven dried at 70°C for 72 hours, and their dry weight measured. The dried roots (excluding nodules) and top were milled through 100 mesh for estimation of total N by the Kjeldahl digestion method.

For determination of yield, plants were raised in 1/5,000a Wagner's pots. Pods with seeds were sun-dried for 15 days after harvest, and the pod number counted. The weight and seed number were also recorded after separation from pod shells.

Except for yield determination (because of poor seed setting in the second trial) there were two trials with four replications. Data were tested for homogeneity of results between the two trials. Data of the repeated trial were pooled for analysis, since variance was homogenous (two-tailed F test, $P=0.05$). The treatment means were separated by Fisher's Least Significant Difference test (LSD, $P=0.05$).

Results

1. Nodulation

Data on nodulation are shown in Fig. 1. A significant reduction of nodule number was observed in plants grown with 1.0% of chitin- or chitosan-amended soil compared to control at both 28 and 42 DAP ($P=0.05$). Although 0.50% chitin treated plants produced a signifi-

Fig. 1. Nodulation of soybean grown in soil amended with different concentrations of chitin or chitosan in comparison to non-amended soil (C) at different days after planting. Vertical bar represents $LSD_{0.05}$.

cantly lower nodule number (73/plant), plants in the same concentration of chitosan produced a statistically similar number (102/plant) to control at 28 DAP. On the other hand, plants grown in 0.10~0.25% chitin- or chitosan-amended soils formed consistently the greatest number of nodules at both harvest dates (28 & 42 DAP).

At sampling day 28, there was a significant ($P=0.05$) reduction of nodule fresh weight of soybean grown in all levels of chitin- or chitosan-amended soil (Fig. 1). Only the plants treated with 0.10% chitin or chitosan produced greater ($P=0.05$) nodule fresh weight at 42 DAP, the other amended treatments still produce lower nodule fresh weight at this harvest date when compared with the control.

The nodule growth was most pronounced between 28 to 42 DAP for 0.10% amended treatment (Table 1). During this period, the average nodule weight increased from 4.57 mg nodule⁻¹ to 12.69 mg nodule⁻¹ for 0.10% chitin, and 7.15 mg nodule⁻¹ to 12.54 mg nodule⁻¹ for 0.10% of chitosan treatments. Although there was a little nodule growth at 0.25% treatments during 28 DAP and 42 DAP, hardly any nodule growth occurred at 1~0.50% chitin or in the chitosan treatments during this period. Nodule growth in non-amended soil was faster than that of chitin- or chitosan- amended soil during the early growth stage. Nodules in nonamended treatment also were more massive, and clustered mostly around crown roots than in the 0.10% chitin or chitosan treatments that formed

Table 1. Influence of different concentrations of chitin- or chitosan- amended soils on nodule growth (mg nodule^{-1}) at different days after planting.

Treatment	Chitin		Chitosan	
	28 DAP	42 DAP	28 DAP	42 DAP
Cont ^{a)}	13.81	14.82	13.81	14.81
0.10%	4.57	12.69	7.15	12.54
0.25%	1.53	4.51	1.39	4.77
0.50%	1.27	3.01	0.91	0.94
1.0%	0.94	0.96	0.69	0.81
LSD _{0.05}	0.97	0.90	1.17	1.90

^{a)} Nonamended soil.

Fig. 2. Acetylene reduction activity of soybean root nodule grown in soil amended with different concentrations of chitin or chitosan in comparison to nonamended soil (C) at different days after planting. Vertical bar represents LSD_{0.05}.

numerous nodules at this growth stage (28 DAP).

2. Acetylene reduction activity

At the first harvest, acetylene reduction activity in root nodules decreased significantly, irrespective of chitin or chitosan concentrations added to the growth medium ($P=0.05$) (Fig. 2). Like the increase in nodule fresh weight, the N₂ fixation activity of root nodules at 42 DAP increased remarkably ($P=0.05$) in treatments where the plants received 0.10% chitin or chitosan. Although there was a substantial increase (6.55 to 30.9 for chitin and 6.35 to 29.0 for chitosan) in ARA value at 0.25% concentrations, this value was still low compared to the lower concentration and control. Acetylene reduction activity at 1.0 ~0.50% treatment showed the lowest value at two stages of plant sampling.

3. Dry matter yield

At the first harvest, total dry matter production in soybean plants was decreased with the increasing concentrations of amendments material in the growth medium (Table 2a & Table 2b). However, at final harvest, both 1% chitin or chitosan amendments produced significantly less dry matter than those of the nonamended control and other amendments. Dry matter production due to amendment with 0.50% chitin was found significantly ($P=0.05$) lower than the control, but this difference, between 0.50% chitosan and control, was not significant. A high dry matter was recorded in 0.10~0.25% chitosan amendment, but plants with the same concentrations of chitin produced dry matter similar to that of control at 42 DAP.

Table 2a. Dry matter production^{a)} of soybean grown in soil amended with different concentrations of chitin in comparison to nonamended soil at different days after planting.

Treatment	28 DAP			42 DAP		
	Top	Root ^{b)}	Total	Top	Root	Total
Cont ^{c)}	3.47	1.31	4.89	8.47	2.79	11.27
0.10%	3.41	1.17	4.49	8.80	2.94	11.74
0.25%	3.25	1.11	4.46	8.56	2.76	11.32
0.50%	2.91	1.09	4.00	7.59	2.49	10.08
1.0%	2.69	1.01	3.69	4.72	1.82	6.54
LSD _{0.05}	0.38	0.17	0.53	0.87	0.61	1.31

^{a)} Mean of two trials each with four replications.

^{b)} Including nodule.

^{c)} Nonamended soil.

Table 2b. Dry matter production^{a)} of soybean grown in soil amended with different concentrations of chitosan in comparison to nonamended soil at different days after planting.

Treatment	28 DAP			42 DAP		
	Top	Root ^{b)}	Total	Top	Root	Total
Cont ^{c)}	3.47	1.31	4.89	8.47	2.79	11.27
0.10%	3.50	1.17	4.61	9.59	3.86	13.40
0.25%	3.45	1.19	4.55	9.09	3.63	12.72
0.50%	3.19	1.20	4.39	7.82	3.04	11.08
1.0%	3.02	1.11	4.12	7.35	2.30	9.66
LSD _{0.05}	0.71	0.12	0.82	0.87	0.37	1.04

^{a)} Mean of two trials each with four replications.

^{b)} Including nodule.

^{c)} Nonamended soil.

Table 3. Total N content of soybean (leaves and stems) grown in soil amended with different concentrations of chitin or chitosan in comparison to nonamended soil at different days after planting.

Treatment	28 DAP		42 DAP	
	Chitin	Chitosan	Chitin	Chitosan
mg N per plant				
Cont ^{a)}	78.60	78.58	140.49	140.49
0.10%	77.66	67.19	146.08	137.97
0.25%	96.35	72.00	170.06	153.65
0.50%	113.56	121.52	171.24	176.70
1.0%	128.48	136.46	192.24	195.34
LSD _{0.05}	39.75	18.92	24.60	18.83

^{a)} Nonamended soil.

4. Nitrogen content

The soybean plants (leaves and stems) accumulated the highest nitrogen levels due to treatment with chitin or chitosan in the range of 1.0~0.50% at the time of both sampling dates (Table 3). However, plants that received 0.25% chitin in the growth medium

showed a slightly higher amount of N at 28 DAP, and a significantly higher amount at 42 DAP compared with nonamended treatment. While the plants treated with 0.10% chitin and 0.10~0.25% chitosan yielded a similar amount of N, as yielded by control at both sampling dates.

Table 4. Yield and yield components^{a)} of soybean grown in soil amended with different concentration of chitin or chitosan in comparison to nonamended soil.

Treatments	Seed yield (g/plant)	Pod no. (per plant)	Seed no. (per pod)	Seed no. (per plant)
Chitin :				
Cont ^{b)}	10.11	41.50	1.45	59.64
0.10%	12.60	46.50	1.43	66.55
0.25%	12.00	42.75	1.37	58.53
0.50	10.24	37.75	1.42	52.99
1.0%	9.35	35.75	1.40	49.69
LSD _{0.05}	1.31	4.99	NS ^{c)}	9.41
Chitosan :				
Cont	10.11	41.50	1.45	59.64
0.10%	13.46	49.50	1.39	69.13
0.25%	13.05	43.75	1.47	64.26
0.50%	12.00	40.50	1.38	55.54
1.0%	10.93	38.50	1.35	51.93
LSD _{0.05}	1.86	4.43	NS	6.57

^{a)} Mean of one trial with four replications.

^{b)} Nonamended soil.

^{c)} Not significant.

5. Seed yield and Yield components

The seed yield of soybean plants was higher in 0.10~0.25% of chitin- or chitosan-treated soil than that of nonamended soil (Table 4). Both pods (19% of control) and seed number (16%) per plant in 0.10% chitosan, while only seed number (12%) in 0.10% chitin treatment increased significantly. However, seed number per pod was not influenced by any of the amendment concentrations.

Discussion

The present study demonstrate that the nodule fresh weight was decreased by addition chitin or chitosan to the soil, while nodule number was not markedly affected, indicating an effect on nodule development rather than root infection at the early growth stage of plants (Fig. 1). This result is like the effect of combined nitrogen in the growth medium¹¹⁾ where nodule fresh weight was greatly reduced compared to nodule number, and the reduction depends on the amount and form of combined nitrogen^{8,19,29)}. Our result also showed a greater reduction of nodule fresh weight when concentration of chitin and chitosan were increased from 0.10% to 1% in growth medium. Kjeldahl analysis shows chitin contained 6.54% N and chitosan

contained 5.9% N. During decomposition of these materials by soil micro organisms, nitrate N might have been released resulting in the inhibition of nodule growth. The higher N content in the plant top (Table 3) due to 1 ~0.5% of treatments reflected the liberation of N from applied chitin or chitosan. Nitrate liberation from the chitin decomposition by micro organisms demonstrated by former investigators^{1,16,28)}. However, the exact reason for the inhibition of nodule growth is not well understood.

Interestingly, nodule in 0.10% treated plants were more numerous in number and distributed in lateral roots from earlier growth stage. At second harvest a consistently higher nodule number and fresh weight was observed in this treatment. This result suggests that low concentration has stimulatory effects on rhizobial multiplication, which might have extended infection to root hairs by the resident rhizobia at the early growth stage, and nodule-tissue development at later growth stage.

Hardarson et al.¹⁰⁾ postulated that nodules clustered around the crown of root are crucial for nitrogen fixation in the early growth stage of development, and the earlier, formed nodules have been reported to suppress further nodule production^{18,24)}. Our results corrobo-

rate their results for soybean raised in nonamended soil. The N_2 fixation activity and nodule fresh weight of nonamended control plants were significantly higher than all other treatments at first harvest. At second harvest, plants treated with 0.10% produced the higher value of N_2 fixation activity. The higher ARA value in this treatment may apparently be due to secondary nodule formation and for the growth of earlier formed nodule. Hardarson et al.¹⁰⁾ used the ^{15}N technique to show both secondary root nodules could even be superior to primary root nodule in N_2 fixation at a later growth stage. On the contrary, very little N_2 -fixation activity was detected with the 0.25~0.5% concentrations.

Specific reduction activity of root nodules was not greatly influenced by different levels of concentration (data have not shown). This indicates that nodule tissues were mainly responsible for ARA value. The regression analysis showed that nodule weight was more closely related to nitrogen fixation than nodule numbers. The corresponding correlation coefficient (r) were 0.98 at 28 DAP and 0.93 at 42 DAP ($P=0.05\%$) for Chitin and 0.99 at 28 DAP and 0.97 at 42 DAP ($P=0.05\%$) for chitosan. These results corroborated with others³⁾.

The dry matter yield of soybean plants grown in nonamended soil was higher at an early grown stage than that of chitin or chitosan treatments. On the contrary, dry matter yield at 0.10% of soil amendment treatments was higher at second harvest. The increased dry matter production in this treatment might be due to increased N_2 fixation. Rabie and Kumazawa²⁷⁾ and Kitou et al.¹⁷⁾ also found higher dry matter production and better growth of soybean plants due to higher N_2 fixation. Although an increased N accumulation in the plant tops at 1.0~0.50% treatments was observed, these plants fixed less nitrogen (Fig. 2), indicating that plants absorbed more soil N possibly from amended materials. In spite of high nitrogen accumulation in these plant tops (Table 3), dry matter production was not increased, which indicated that this concentration had not only suppressive effect on root nodulation and nitrogen fixation but also a suppressive effect on soybean growth. Some toxic volatilized substances might have been responsible for the poor

growth performance of soybean plants at the above concentrations. The growth of rice seedling was noted to be suppressed by 1~2% chitin in growth medium²⁰⁾.

The higher pod and seed number and total seed yield in 0.10% treated plants was surmised possibly due to increased N_2 fixation in soybean. Harada et al.⁹⁾ also noted similar results in chitosan-applied soybean.

Overall, these data indicate that irrespective of amendment levels used, chitin or chitosan had a deleterious effect on nodule tissue development and N_2 fixation in soybean plants at early growth stages. However, the use of low concentration (0.10%) of these materials in growth medium enhanced nodulation, N_2 fixation, and better plant growth in comparison to control at later growth stage soybean. The soybean seed yield was also increased slightly at this level of chitin or chitosan amendment.

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** In Russian with English summary.