ENHANCEMENT OF THE BIOLOGICAL NITROGEN FIXATION OF SOYBEAN PLANTS INOCULATED WITH *BRADYRHIZOBIUM JAPONICUM*

By

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ABSTRACT

Biological nitrogen fixation of soybean plants inoculated with *Bradyrhizobium japonicum* was evaluated using bio-organic fertilizers including rice straw compost and its tea, rice bran and its aqueous extract individually or in combinations. Under static culture conditions, rice bran was used as substrate by some selected PGPR strains which reached their maximum viable counts *in vitro* after 72 h of incubation. Due to use of 0.25 g L^{-1} rice bran, specific growth rate (μ) of *Azotobacter chroococcum* reached its maximum value (0.017 h^{-1}), but it decreased to 0.013 h^{-1} by using of 0.50 g L^{-1} rice bran. For *Azospirillum brasilense*, no inhibitory effect of rice bran was observed and the culture gradually reached its maximum μ-value (0.021 h^{-1}) as a result of 0.50 g L^{-1} rice bran.

Under greenhouse conditions, soil supplemented with compost and its grown plants inoculated with *B. japonicum* as well as foliar sprayed with both compost tea and rice bran extract was the most effective (superior) treatment in comparison with the other applications. Number of nodules per plant was increased to 47.33 and 33.33 due to the superior treatment in comparison with 14.33 and 16.00 for the inoculated plants with *B. japonicum* alone during 2007 and 2008 seasons, respectively. Due to this treatment, nodular dry weights were approximately as twice as those treated with *B. japonicum* alone. Accordingly, remarkable increase in the dry weight of roots, shoots and seeds was obtained. Superiority of this treatment was also extended to reach the greatest mean values of nitrogen (3.38 and 6.91 %) and phosphorus (0.39 and 0.51 %) in the shoots and seeds, respectively.

Key words: Soybean, Compost, Rice bran, PGPR, *B. japonicum*.

INTRODUCTION

Soybean is one of the most economic legume crops of the world. Since it is rich in oils (17-35 %) and protein (24-45 %), it is considered suitable for feeding humans as well as animals (Ibrahim and Kandil, 2007). It was also described as a strong supplier of soil nitrogen to the subsequent crops. In most cultivated areas of Egypt, soybean is subjected to some problems including reduction of nodule formation. This problem adversely affects its biological nitrogen fixation and consequently the plant growth. The need for suitable applications to overcome these disorders is necessary. Bio-organic fertilization lends itself as practical, environmental-friend, low coast technique to manage and convert biodegradable wastes into something useful. This technique enhances the utilization of biomass wastes of agriculture.

Compost and its tea are the most promising bio-products recently responsible for developing different management programs as plant pest, disease and fertility (Sheuerell and Mahaffee, 2002). Composted wastes or their extracts, produced from local materials such as rice straw, contain such effective microorganisms (EM), or their metabolites, acted as plant growth promoters (Brinton, 1995 and McQuilken *et al.* 1994). In addition, rice-bran is a by-product of the rice milling industry. It has a high nutritive value due to its higher concentrations of vitamin E, B-group vitamins, gamma-oryzanol, rice bran saccharide, and protein (Schramm *et al.* 2007). One of the more recent applications of rice bran is to exploit its eco-friendly nature for the extraction of plant oils instead of hexan which has been known as hazardous organic solvent (Sumantha *et al.* 2006). Therefore, the present study deals with the potential use of rice bran to induce more activation of some selected plant growth-promoting rhizobacteria (PGPR) which enriched the rice straw compost for enhancing N_2-fixation and growth of soybean plants inoculated with its suitable rhizobia (*Bradyrhizobium japonicum*).

MATERIALS AND METHODS

Microorganisms:

Some selected strains of the plant growth-promoting rhizobacteria (PGPR) i. e. *Azotobacter chroococcum, Azospirillum* *brasilense, Paenibacillus polymyxa* and the soybean rhizobial strain (*Bradyrhizobium japonicum*) coded 1577110 were kindly obtained from the stock culture collection of Biological Nitrogen Fixation Unit, Dept. of Soil Microbiology at Sakha Agric. Res. Station, ARC.

Growth estimation :

To investigate the effect of rice-bran application on growth of the bacterial strains, viable counts of the PGPR were carried out using the quantitative plating methods. For cultivation of *Azotobacter chroococcum*, Ashby's medium was used (Allen, 1959). The lactate medium (Dobereiner, 1978) was used for growing *Azospirillum brasilense*. Nutrient broth was the required medium for *P. polymyxa*. Erlenmeyer flasks containing media and rice bran were sterilized, inoculated with each strain separately and incubated at 28 °C. Two rice-bran concentrations of 0.25 and 0.50 g L^{-1} were tested. Flasks free from rice bran acted as control. 0.5 ml (7 days old cultures) for each strain was spread on agar plates of each medium by using driagalsky triangle. The plates were incubated at 28-30 °C and examined daily. Three replicates were used for each strain. Viable counts at 24, 48, 72 and 96 hrs incubation were calculated as follows:

Viable count = No. of colonies x dilution factor x volume of inoculum

Preparation of standard inocula:

For preparation the rhizobial inocula, Erlenmeyer flasks holding 200 ml yeast manitol broth medium (Minamisawa, 1989) were sterilized and incubated by the tested strain. Incubation was carried out on rotary shaker at 28 °C for 7-9 days. The count of rhizobial cells was adjusted to be 10^8 cells / ml using a Petroff-Hauser chamber. Nodulation on soybean (*Glycin max* (L.) Merill. cv. 111) by this strain was confirmed in an inoculation test.

Greenhouse experiment:

This investigation was carried out in experimental pots during two successive seasons of 2007 and 2008 in the greenhouse of Sakha Agricultural Research Station. Seeds were sown in pots of 30 cm in diameter filled with 6 kg clay soil. After germination, seedlings were thinned to three plants per pot. One week after germination, seedlings were inoculated by pipetting 5 ml of its rhizobial inoculum (*B. japonicum*) into the soil around the base of each seedling.

Treatments and determinations:

Rice straw compost, rice bran and/or their extracts were conducted as the main treatments in this study. Composting of rice straw was carried out in the presence of *Trichoderma viridi* (cellulose decomposer) to accelerate the decomposition rate. To enrich the compost with available nutrients, *A. chroococcum, A. brasilense* and *P. polymyxa* were used as selected plant growth-promoting rhizobacteria (PGRP) according to Badawi (2003). After maturation, enriched compost was soaked in distilled water for about 1 kg : 10 L and then filtered to obtain its tea. Three months after composting, chemical and biological properties of the supplemented compost were found to be in **Table 1**.

Table 1. Chemical and biological characters of the supplemented compost.

Character	Value
pH	7.35
EC (ds m^{-1} at 25 °C)	4.98
C/N ratio	16.49
Organic carbon (%)	22.26
Total nitrogen (%)	1.35
Cross seed germination teast (%)*	92.40
Total count of bacteria (cfu/ml)	1.3×10^7
Total count of fungi (cfu/ml)	2×10^5
Total count of actinomycetes (cfu/ml)	5×10^5

* Cross germination test was carried out using *Eruca sativum* seeds after 72 h.

Rice bran was obtained from a local market. Adachi *et al.* (2005) stated that rice bran contains 13.5 % moisture, 13.2 % protein, 18.3 % lipid, 46.1 % carbohydrate (glucide 38.3 % and fiber 7.8 %) and 8.9 % ash. The following treatments were carried out:

a) Untreated plants (control).
b) Plants inoculated with *B. japonicum* alone.
c) Plants inoculated with *B. japonicum* and soil supplemented with compost.

d) Plants inoculated with *B. japonicum* and sprayed with compost tea.
e) Plants inoculated with *B. japonicum* and soil supplemented with compost and rice-bran in combination.
f) Plants inoculated with *B. japonicum* and sprayed with compost tea as well as soil treated with rice-bran.
g) Plants inoculated with *B. japonicum* and sprayed with compost tea as well as soil supplemented with compost.
h) Plants inoculated with *B. japonicum* and sprayed with rice-bran aqueous extract as well as soil supplemented with compost.
i) Plants inoculated with *B. japonicum* and sprayed with rice-bran aqueous extract and compost tea in combinations as well as soil supplemented with compost.

For soil application, 50 g maturated compost and/or 2.5 g rice-bran and/or their combinations were supplemented per pot. As foliar spray, diluted extracts of 10 % compost tea and/or 0.1 % rice-bran tea and/or their combinations were regularly applied every 15 days during the vegetative growth stage. Each treatment was represented by four replicates. Pots were kept in greenhouse and watered when even required. The cultural practices, i.e. fertilization, irrigation and pest control were also carried out.

After 60 days of planting, plants were uprooted. The nodulation status was evaluated by determination of the following parameters:

a) Number of nodules per plant.
b) Dry weight of nodules, roots and shoots (g/plant).
c) Total nitrogen and phosphorus of shoots (%).

After 120 days of planting, yield parameters were determined as follows:

a) Dry weight of seeds (g/plant).
d) Total nitrogen and phosphorus of seeds (%).

Total nitrogen and phosphorus contents in the shoots and seeds were determined as described by Jackson (1973).

Statistical analysis:

Data were statistically tested for one-way analysis of variance (ANOVA) using SPSS computer software program and Duncan's multiple range tests were applied for comparing means (Duncan, 1955).

RESULTS AND DISCUSSION

Microbial activity:

Effect of rice bran application on viable count of the applied rhizobacteria (PGPR) such as *Azotobacter chroococcum* and *Azospirillum brasilense* during the incubation periods were investigated. For *Azotobacter chroococcum* (**Fig. 1**), the culture was rapid grown due to use both of 0.25 and 0.50 g L^{-1} rice bran. Maximal count of the viable bacteria was obtained after 72 hrs from inoculation due to use of 0.25 g L^{-1} rice bran, indicating higher metabolic activity in comparison with the other concentration. On the other hand, the culture free from rice bran took a considerable long time before reaching its maximum growth, indicating slower induction of adaptive enzymes at the lag-phase. At 96 h incubation, all cultures exhibited sharp decrease in their viable count, indicating occurrence of the decline phase.

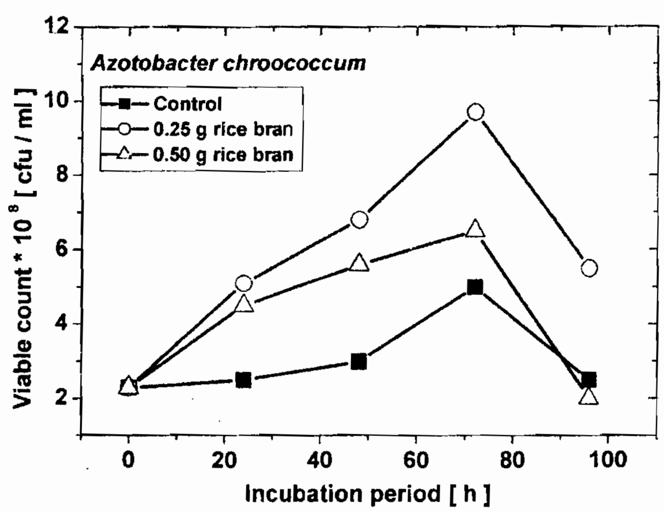

Fig. 1. Viable count of *Azotobacter chroococcum* during different incubation periods.

For *Azospirillum brasilense*, viable counts versus the incubation periods are given in **Fig. 2**. It illustrates that application of 0.5 g L^{-1} rice bran was the most effective concentration for producing higher viable count of 17 x 10^8 cfu/ml after 72 hrs of incubation in comparison with the lower concentration. It indicates that *A. brasilense* has a great potential to consume more concentration of rice bran. On contrary to the other strain, the untreated culture (control) of *A. brasilense* did not need to lag-phase before reaching its accelerating phase, indicating well adaptation. Therefore, no growth limitation of *A. brasilense* was obtained under the investigated conditions. Data indicate also that, log phase of both *A. chroococcum* and *A. brasilense* was immediately occurred after rice bran application, indicating fast induction of the enzymes to produce higher biomass yield.

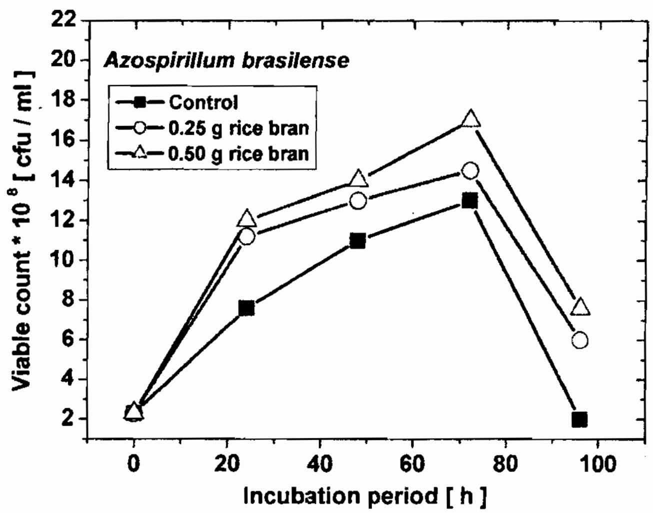

Fig. 2. Viable count of *Azospirillum brasilense* during different incubation periods.

Data obtained from cultivation of both strains indicate a great potential of the role played by the catabolic enzymes of rice bran, fast induction by *A. brasilense* but slow with *A. chroococcum*. Rice bran was suggested to be used as a substrate for enzyme production in several microorganisms such as lipase (Rao *et al.* 1993), glucoamylase (Tani *et al.* 1986) and protease (Sumantha *et al.* 2006), leading to acceleration of the bacterial growth. After 72 hrs incubation, reductions of the viable counts were probably due to substrate limitation and/or production of inhibitors such as metabolites leading to the decline phase. Tanaka *et al.* (2006) stated that D-lactic acid was the main metabolite produced by rice bran biodegradation which probably responsible for suppressing the bacterial growth.

From these results, a basic question rises: will further increase in rice bran concentration be necessary to obtain more biomass activities? Therefore, specific growth rate (μ) of each strain was calculated according to Shalaby (2003) as follows:

$$\mu = (C_{X\,t} - C_{X\,0}) / ((t_t - t_0) * C_X)$$

Where:

μ	= Specific growth rate	$[h^{-1}]$
$C_{X\,t}$	= Biomass concentration at time t	[cfu/ml]
$C_{X\,0}$	= Biomass feed concentration	[cfu/ml]
C_X	= Average of biomass concentration	[cfu/ml]
t_t	= Running time	[h]
t_0	= Start time	[h]

The resulting μ–values after 72 h of incubation versus rice bran concentrations are plotted in **Fig 3**.

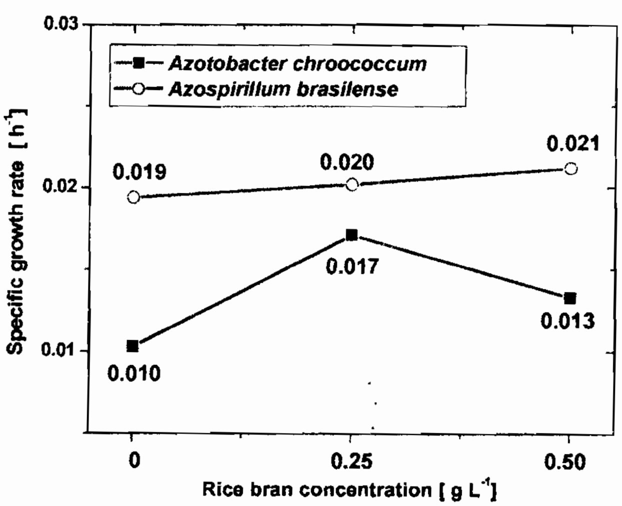

Fig. 3. Specific growth rate (μ) of both *A. chroococum* and *A. brasilense* as functions of rice bran concentration after 72 hrs of incubation period.

Data indicate slow development in the μ-values for *Azospirillum brasilense*, or may tend to be constant, due to use of 0.25 and 0.50 g L^{-1} rice bran. However, the specific growth rate was ranged between 0.020 and 0.021 h^{-1} for both concentrations, respectively. Therefore, *A. brasilense* was able to grow on a further increase of rice bran concentration with more biomass formation. On contrary, growth of *Azotobacter chroococcum* was inhibited by using of 0.50 g L^{-1}rice bran. Specific growth rate was decreased from 0.017 to 0.013 h^{-1} by using of 0.25 and 0.50 g L^{-1} rice bran. It means that increasing rice bran to a concentration more than 0.25 g L^{-1} will inhibit the specific growth rate of *A. chroococcum*, indicating production of inhibitory metabolite. Lactic acid resulted from rice bran exhibited a wide spectrum of growth inhibition with various bacterial strains (Yamamoto *et al*., 2003). Therefore, utilization of the lower rice bran concentration was more effective to obtain higher specific growth rate of the selected PGPR strains.

Symbiotic N_2-fixation:

Data in **Table 2** show the effect of single and/or mixed treatments of compost, rice-bran and their extracts on the N_2-fixing parameters of soybean plants inoculated with *B. japonicum*. There was a positive effect of all treatments in comparison with the control, which could be attributed to the enhancement of nodule formation by *B. japonicum*. The results were more pronounced in the pots inoculated with *B. japonicum*, compost and foliar spray using combination of rice-bran aqueous extract and compost tea as the superior treatment. Nodule numbers were increased to 47.33 per plant and the nodular dry weight to 0.51 g/plant during the first season. While, the corresponding values of only rhizobial inoculated treatment showed 14.33 nodules/plant and 0.26 g/plant of nodule dry weight. Similar positive effect was also observed during the second season, indicating the enhancing role of compost, compost tea activated with rice bran aqueous extract on indigenous rhizobia in plant rhizosphere. The control plants were nod void of nodules, indicating the well known least activity of the native rhizobia. It has been proved earlier that there is a relationship between the mass of effective nodules and quantity of nitrogen fixed (Badr, 1984). Co-inoculation of soybean with *B. japonicum* and some PGPR, improved root nodules, number or mass, and increased the nitrogenase activity (Dashti *et al.* 1997). Dashti *et al.* (1998) confirmed an enhanced soybean nodulation and increased nitrogen-fixing activity in the presence of the plant growth promoting rhizobacteria (PGPR).

Table 2. Effect of compost, rice-bran and their teas on numbers and dry weight of nodules in soybean plants inoculated with *Bradyrhizobium japonicum*.

Treatments	2007		2008	
	No. of nodules /plant	D. w. of nodules g/plant	No. of nodules /plant	D. w. of nodules g/plant
Control	0.02 a	0.002 a	2.33 a	0.004 a
R	14.33 b	0.26 c	16.00 c	0.11 bc
R+C	15.67 bc	0.35 d	18.67 d	0.12 c
R+Ct	13.33 b	0.35 d	14.00 c	0.13 d
R+C+Rb	13.67 b	0.17 b	10.00 b	0.11 b
R+Ct+Rb	23.33 d	0.29 c	31.00 f	0.20 f
R+C+Ct	20.33 cd	0.38 d	22.30 e	0.18 e
R+C+Rbt	36.33 e	0.46 e	23.00 e	0.19 f
R+C+Ct+Rbt	47.33 f	0.51 e	33.33 f	0.20 f
L. S. D 5 %	5.14	0.05	2.64	0.01

Means followed by the same letter are not significantly different at the 5% level of probability. R = *Bradyrhizobium japonicum*; C = Compost; Ct = Compost tea; Rb = Rice bran; Rbt = Rice bran aqueous extract. D. w = Dry weight.

Vegetative growth:

The positive effect of the superior treatment was extended to the vegetative growth parameters i. e. dry weight of roots, shoots and seeds were examined and data are given in **Table 3**. Significant increases were achieved in the dried tissues due to the superior treatment in comparison with the other application types, indicating higher metabolic activity. Data indicate that the greatest dry weight values were recorded in the presence of rice bran aqueous extract, not with rice bran. It is clear that rice bran aqueous extract is the suitable form for growth activation. It means that solubility of rice bran is necessary to produce the active ingredients and nutritional substances required to enhance the plant growth in comparison with rice bran as a row material. This could be attributed to an increase of the nitrogen content of plants as a result of symbiotic N_2-fixation activity beside the role of the bio-organic fertilization.

Table 3. Effect of compost, rice-bran and their teas on dry weight of shoots, roots and seeds of soybean plants inoculated with *Bradyrhizobium japonicum*.

Treatments	Dry weight (g /plant)					
	shoot	root	seeds	shoot	root	seeds
	2007			2008		
Control	13.49 a	4.59 a	7.23 a	18.03 a	4.07 a	8.15 a
R	27.32 d	7.17 b	10.42 c	28.03 c	6.17 c	12.11 d
R+C	27.20 d	8.22 bcd	12.28 efg	18.84 a	8.32 f	13.13 e
R+Ct	22.18 c	7.46 bc	11.70 de	25.37 b	7.63 e	12.09 d
R+C+Rb	17.20 b	5.48 a	9.18 b	18.87 a	5.16 b	9.10 b
R+Ct+Rb	26.45 d	7.63 bc	12.20 ef	25.77 bc	8.31 f	12.14 d
R+C+Ct	23.59 c	7.59 bc	11.43 d	26.05 bc	7.05 d	11.07 c
R+C+Rbt	27.59 d	8.43 cd	12.38 fg	25.38 b	7.74 e	13.25 e
R+C+Ct+Rbt	28.17 d	9.16 d	12.85 g	27.97 c	8.77 f	13.84 f
L. S. D 5 %	1.83	0.97	0.57	2.17	0.44	0.22

Means followed by the same letter are not significantly different at the 5% level of probability. R = *Bradyrhizobium japonicum* ; C = Compost; Ct = Compost tea; Rb = Rice bran; Rbt = Rice bran aqueous extract.

Therefore, results obtained herein indicate enhancement of the metabolic activity via exploiting the interactions between rice bran solution and compost tea with their high nutritional value (Emino and Warman, 2004), and rhizobial inoculation that can enrich the plant and soil with N_2 element (Biswas *et al*., 2000). Therefore, activated compost tea by rice bran tea could be considered as an effective bio-fertilizer, consisting of essential components required for cell division and elongation due to being enriched in macro-and microelements, vitamins and phytohormones. To increase growth and yield of cherry tomato, rice bran mulching was also applied by Rodriguez (2007).

N and P contents:

The positive effects of the co-inoculation treatments were also extended to the total nitrogen accumulation (**Fig. 4a**) and phosphorus (**Fig. 4b**) contents in soybean shoots and seeds. The same pattern was obtained, average data of both seasons were considered. This refers to accumulation of both N and P in the seeds more than in the shoots, indicating transport and storage both elements in the reproductive organs.

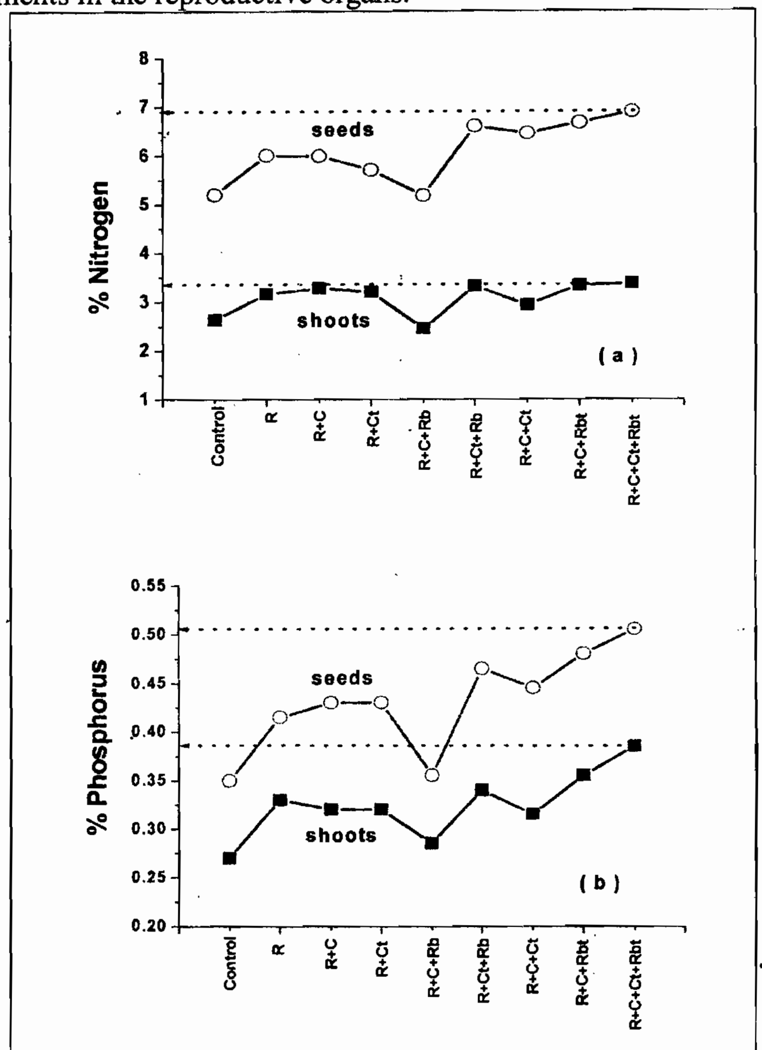

Fig. 4. Effect of compost, rice-bran and their teas on nitrogen **(a)** and phosphorus **(b)** contents in shoots and seeds of soybean plants inoculated with *B. japonicum*. R = *Bradyrhizobium japonicum*; C = Compost; Ct = Compost tea; Rb = Rice bran; Rbt = Rice bran aqueous extract.

The highest N and P contents in the shoots and seeds were obtained also via the integrated treatment including rhizobia, compost, compost tea and rice bran aqueous extract (superior treatment). On the contrary, a remarkable decrease in N and P contents in the control plants was achieved, indicating sever reduction in the metabolic activity. All other treatments showed positive effect and achieved intermediate values between those two extremes. According to Matiru and Dakora (2004), rhizobia naturally produce many compounds i. e. IAA, cytokinins, absicic acids, lipo-chito-oligosaccharides, vitamins and rhiboflavin. These molecules promote plant growth and colonization of rhizobia and their adaptation in soybean roots would be enhanced to increase plant development. Fertilization with compost or compost tea with their high nutritional values those are present in soluble chemical components into an aqueous sphere (Dazzo and Yanni, 2006). Moreover, nutritional value of compost or compost tea was strongly enhanced in the present work due to co-inoculation using rice bran aqueous extract or at least with rice bran. Due to its high nutritional value, rice bran was used by Rodriguez (2007) to mulch a surface of tomato field and enhance the vegetative and yield properties of cherry tomato plants.

Therefore, our data indicate that growth of soybean plants could be enhanced and consequently improved through biological N_2-fixation and fertilization with compost activated with rice bran and their teas. In growth promotion, rhizobial inoculation can fix appreciable amounts of N_2 and enrich the plant and soil with this important nutrient (Sprent and Sprent, 1991) and increase nutrient uptake and P solubilizer (Biswas *et al.* 2000). Moreover, Dakora (2003) reported that rhizobia have a great potential for promoting growth of the host plant. Therefore, the present study presents a new compost activator that enhances the decompositions process by increasing the population of beneficial organisms, resulting in high quality compost. The postulated mechanisms of plant growth stimulation by associative bacteria are stimulation of root growth by production of phytohormones (Bothe *et al.* 1992 and Kloepper *et al.* 1980).

In conclusion, this investigation presents rice bran, or its aqueous extract, as an important organic substance which possesses high potential to produce active ingredients required for enhancing decomposing process of the agricultural wastes. This was extended to increase the growth rate of the beneficial microorganisms enriched with the compost.

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الملخــص العربـــى

تنشيط التثبيت الحيوى للنيتروجين فى نباتات فول الصويا الملقحة بواسطة *Bradyrhizobium japonicum*

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تـم تقيـيم عمليـة التثبيـت الحيـوى للنيتـروجين فـى نباتـات فـول الصـويا الملقحة بريزوبيا *Bradyrhizobium japonicum* بإستخدام أسمدة حيويـة - عضوية تشمل كمبوست قش الأرز وكذلك رجيع الأرز ومستخلصاتهم بصورة فردية أو على هيئة خليط . فقد أستخدم رجيع الأرز كمادة نمو لبعض السلالات البكتيرية PGPR تحت ظروف المزرعة الأستاتيكية والتى وصلت من خلالها الأعداد الحية للخلايا الى أقصاها بعد ٧٢ ساعة تحضين فى المعمل . وقد وصل معدل النمو النوعى (μ) لبكتيريا *Azotobacter chroococcum* إلى قيمته القصوى (٠,٠١٧/ ساعة) عند إضافة ٠,٢٥ جم/ لترمن رجيع الأرز، ولكن حدث إنخفاض فى قيمة μ عند تركيز ٠,٥٠جم/ لتر . وبالنسبة لبكتيريا *Azospirillum brasilense* فلم يلاحظ أى تـأثير تثبيطى لرجيع الأرز عاى نموه ، وبلغت المزرعة أقصى معدل نمو نوعى (٠,٠٢١/ ساعة) نتيجة لإضافة ٠,٥٠ جم/لتر من رجيع الأرز .

أمـا تحت ظروف الصوبة ، فإن معاملـة النباتـات الملقحـة ببكتيريـا *B. japonicum* والناميـة فـى تربـة مخلوطـة بالكمبوسـت ورش مجموعهـا الخضرى بخليط من مستخلصى (شاى) الكمبوست ورجيع الأرز كانت ألأكثر تـأثيرا (المتفوقـة) بالمقارنـة بالمعـاملات الأخـرى . وقـد تزايـدت أعـداد العقـد البكتيرية لجذورالنبات الواحد الى ٤٧,٣٣ و ٣٣,٣٣ نتيجة للمعاملـة المتفوقة مقارنة بـ ١٤,٣٣ و ١٦,٠٠ عقدة للنباتـات الملقحـة ببكتيريـا *B. japonicum* وحدها خلال موسمي ٢٠٠٧ و ٢٠٠٨ م على التوالى . وتبعا لهذه المعاملـة ، فقد تضاعف الوزن الجاف للعقد الجذرية بمقارنتها بالمعاملة بالريزوبيا فقط دون أى أضافات . وبنـاء على ذلك ، فقد زاد الوزن الجاف للجذور والمجموع الخضرى والبذور بوضوح . وبسبب تفوق هذه المعاملـة قد بلغت متوسطات القـيم الـى أقصـاها للنيتـروجين (٣,٣٨ و ٦,٩١ %) و الفسـفور (٠,٣٩ و ٠,٥١ %) فى المجموع الخضرى والبذور على التوالى .