JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4393-4406

Effect of Plant Growth Regulator GA_3 and PIX on Cell Growth and Structure of *Rhizobium fredii* and *Bradyrhizobium japonicum*

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(Received: 11 November 2013; accepted: 15 January 2014)

A comparative study was conducted on the effects of different concentrations (10^{-1}-10^{-6}) of the plant growth regulator GA_3 and PIX on the bacterial population, growth rate, pH, and microstructure of *Rhizobium fredii* (GIM 1.227) and *Bradyrhizobium japonicum* (GIM 1.94). The results showed that the bacterial population, growth rate, pH value and relative expression of *nod*A and *nod*D of *R. fredii* and *B. japonicum* were increased, accelerated, reduced and up-regulated respectively, when the GA_3 concentration in the medium was 10^{-3}; however, the bacterial population, growth rate, pH value and relative expression of *nod*A and *nod*D of *R. fredii* and *B. japonicum* were decreased, decelerated, increased and down-regulated respectively, when the PIX concentration in the medium was 10^{-2}. The microstructure of *Rhizobium* was altered by the plant growth regulator.

Key words: *Rhizobium fredii*, *Bradyrhizobium japonicum*, Plant growth regulators, character of *Rhizobium*, *nod* gene.

Plant growth regulators (PGRs), exogenously applied to improve yields, stress resistance, and overall fruit quality have become increasingly important in modern agricultural production. The more common PGRs include Gibberellins (GA_3), Mepiquat chloride (PIX), Indoleacetic acid (IAA) and Cytokinins (CTK)[1, 2]. Foliar applications of GA_3 are often used in various crops to stimulate growth and development with the plant[3]; Cell, stem, and stalk elongation[4]; leaf enlargement[5]; early crop maturation[6], breaking of seed dormancy, seed germination[7]; leaf abscission and increases in dry weight and yield[8] are common examples of the effects of GA_3. Conversely, PIX is most often used as a growth retardant, inhibiting cell elongation, weakening terminal buds, and reducing overall plant vigor. It has also been demonstrated to affect leaf coloration, photosynthetic capacity, tolerance to drought, cold, and saline-alkali soils[9-11]. Morandi et al. observed decreased soybean stem dry weight in plants exposed to PIX and CCC, whereas PIX alone increase dry matter partitioning to seeds[12].

Beginning in the 1980s, mutagenesis experiments using pea (*Pisum sativum*) produced abnormal nodulation phenotypes including

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nonnodulating, poorly nodulating, and hypernodulating mutants[13]. Present studies have indicated that the nodulation process is regulated by several plant growth regulators. Also, genetic analysis has revealed that a gain-of-function mutation in a cytokinin receptor promotes spontaneous nodule formation. On the other hand, ethylene (ET), abscisic acid (ABA), jasmonate acid (JA), salicylic acid (SA) and brassinosteroid (BR) were shown to negatively affect nodule formation[14]. Ferguson et al. demonstrated that in pea mutants that are deficient in GA biosynthesis, nodule formation is aborted, and is restored on the application of exogenous GA, although the addition of higher concentrations of GA no longer restores nodule formation in these mutants[15]. But the application of higher concentrations of GA also suppresses nodulation in wild-type plants. Maekawa et al. from their study results, conclude that GA inhibits the nodulation signaling pathway downstream of cytokinin, possibly at NSP2, which is required for *Nod* factor-dependent NIN expression[16]. In soybean, Bano and Harper found that although exogenous ABA actually decreases nodule number in both the wild type and a supernodulation mutant, it also adversely affects plant growth[17]. Suzuki et al. also found that lower-than-normal concentrations of endogenous ABA enhance nodule formation[18]. So they hypothesize that the ABA concentration controls the number of root nodules.

Nodulation (*nod*) genes, which located in the sym-plasmid of *Rhizobial*, the major function were form and mutual exchange of diffusible signal molecules at initial of symbiosis of plant-nodules. It was including that the common *nod* genes (*nod*ABC), regulatory (*nod*D) and the host specificity of nodulation (*hsn* genes), e.g. *nod*H. There into, common *nod* genes (*nod*ABC) with highly homology are functionally interchangeable between different *Rhizobial* species, and they are required to elicit the curling of plant root hairs and division of meristematic cells. The reason why *nod*ABC are named common *nod* gene is because they are structurally conserved, and the function of host will not be changed by *nod*ABC exchanged during each *Rhizobial* species[19, 20]. If *nod*ABC genes inactivation, the induction of mutualistic symbiosis on plant of *Rhizobial* will be lost, including the curling of plant root hairs, the formation of infection threads, division of meristematic cells and the formation of root nodules[21].The *nod*D gene is transcribed divergently from the *nod*ABC operon[22]. Beside this operon, *nod*D activates the transcription of all three other nodulation operons[23]. The lack of *nod* gene activation in the Sym-strain, and the rescue of *nod*ABC gene expression upon the re-introduction of the *nod*D gene, confirm that *nod*D is nevertheless essential for the transcriptional activation of common *nod* genes[24]. The most important nodulation genes *nod*ABC and *nod*D, as well as the main promoter region between them, had been analyzed in details.

Although much work has been conducted with use of compounds in leguminous crops, the treatment of PGRs is daily foliar application or soaking seeds in most research. Therefore, it is not rule out that the formation of root nodule was affected by root exudates such as some nod factors. Little is known about PGRs effects on bacterial colony-developing, *nod* gene-expressing and cell morphology-changing of free-living cultures of *Rhizobial* bacteria unassociated with these crops. Kosenko et al. reported that 0.1% D1 (a synthetic analog of phytohormones) suppressed bacteria growth; however, different bactozole (compounds of bacterial origin) concentrations (0.001, 0.01, and 0.1%) exerted similar effects on the growth of bacteria when grown in low nitrate concentrations (6 mM)[25, 26]. Brewin also provided some evidence of the role fairly conventional plant growth regulators have on nodule structure[27]. Zahir et al. imply that supplementing *Rhizobial* inoculation with L-Tryptophan could be a useful approach for improving growth, nodulation and yield of mung bean[28]. Cooper suggested that some flavonoids act as antagonists (anti-inducers) of *nod* gene transcriptional activation that is triggered by inducing flavonoids[29]. Thus, the ensuing level of *nod* gene induction is the result of both stimulatory and inhibitory effects. So whether the PGRs with the same effect on flavonoidsÿThese data suggest the importance of further exploring the role such compounds have in host-bacterium relationships in leguminous crops. To understand such, we need to specifically determine the direct effect such compounds have on the bacterium.

MATERIALS AND METHODS

Rhizobium strains and plant growth regulators

In this study, we used two *Rhizobial* strains, *R. fredii* (GIM 1.227) and *B. japonicum* (GIM 1.94), from the ACCC (Agricultural Culture Collection of China), and two plant growth regulators, gibberellins (GA_3) and mepiquat chloride (PIX), from our laboratory.

Reagents

YMA liquid medium: 10.0 g mannitol, 3.0 g yeast, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.25 g K_2HPO_4, 0.25 g KH_2PO_4, titration with DI water to 1000 ml, final pH between 6.8 and 7.0, sterilize at 121° for 20 min.

YMA solid medium

YMA liquid medium plus 2% agar.

GA_3 mother solution

10.0 g GA_3 titration with absolute ethyl alcohol to 100 ml, store at 4°C until use.

PIX mother solution

10.0 g PIX titration with sterile water to 100 ml, store at 4°C until use..

Effect of plant growth regulator in different concentrations on bacterial population of Rhizobial

Concentrations of plant growth regulator GA_3 and PIX mother solution at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} v/v, respectively, were added to YMA solid medium. Aliquots of bacterial solution of *R. fredii* and *B. japonicum* were withdrawn 10^{-4} times. Then the 50-µl diluent was spread on YMA solid medium with different concentrations of GA_3 and PIX and cultured at 28!. Effects of plant growth regulator on bacterial population of *Rhizobial* were determined by the colony counting method when bacterial population of plates appeared evident for about 3 d. An SPSS significance test was performed. Each treatment was repeated three times.

Effect of plant growth regulator on cell growth and pH of Rhizobial

Concentration of plant growth regulator GA_3 and PIX mother solution at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} v/v were added to YMA liquid medium. Then 5% aliquots of bacterial solution of *R. fredii* and *B. japonicum* were inoculated and cultured on sterilized 40 ml of YMA liquid medium with GA_3 and PIX of different concentrations at 28! using a 170-rpm shaker. The cell growth and pH of *Rhizobial* were determined by spectrophotometer VIS-723G at 600 nm and compact pH meter B-212 (NORIBA) at time points as follows: 2 h, 4 h, 8 h, 16 h, 24 h, 36 h, 48 h, 72 h, and 96 h. Each treatment was repeated three times.

Effect of plant growth regulator on nodA and nodD expression of Rhizobial

All H_2O and solutions were treated overnight with 0.1% diethyl pyrocarbonate and autoclaved. All glassware was baked for 12 h at 180!. All plasticware was soaked for 12 h in 3% H_2O_2 and rinsed with H_2O. Total RNA was isolated with the RNeasy Mini kit (QIAGEN, Hilden, Germany) and the concentration of total RNA was quantified at OD_{260}. Using equal amounts of total RNA, cDNA was synthesized with a TransScript a!First Strand cDNA SynthesisSuperMix (TRANSGEN, China): cDNA was made from 8 µl total RNA (1000 µg/µl) in a volume of 20 ?l containing 1 µl oligo (dT18), 10 µl 2×TSa!RT Reaction Mix and 1 µl TSa!RT Enzyme Mix for centrifuging 30 s followed by 30 min at 42°C and 5 min at 85°C. The PCR reactions were performed with 2 µl of the cDNA solution in 0.5 µl primer (10 µM) and 6.25 µl Eco GreenI fluorochrome MIX (TRANSGEN) in a total volume of 12.5 µl. The R. fredii 16S rDNA V3 region gene which was amplified by universal oligonucleotide primer (338F, 518R) was used to normalize levels. The primers used for real-time PCR are listed in Table 1. Real-time PCR (95! for 5 min, 40 cycles at 95! for 15 s, 55! for 30 s) was performed with ABI PRISM 7000 (Applied Biosystems, USA). The ΔΔCT (threshold cycle) method of comparing expression data was applied and the relative quantitative value was expressed as $2^{-\Delta\Delta CT}$. The specificity of the amplification was confirmed by the presence of a single peak in a dissociation curve at the end of the PCR reaction. All reactions were done in triplicate. Data were analysed with the software SDS version 2.3 (Applied Biosystems).

Effect of plant growth regulator on microstructure of Rhizobial

YMA solid media with *R fredii* and *B japonicum* colonies were first excised and trimmed to approximately 10 mm × 10 mm specimens (1-2 mm, as thin as possible). Each specimen was fixed in a solution containing (%, v/v): 2.5 glutaraldehyde; filter-sterilized seawater, 85; and distilled water, 15. After fixation the specimen was washed repeatedly with double-distilled sterile water to remove salt crystals. The specimen was

dehydrated in a graded ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 96%, each change 5 min) and subsequently in HMDS (hydroxymexamethyldisilazane). After drying, the specimen was attached to scanning electron microscope (SEM) stubs using double-sided conductive tape and sputter-coated with gold. The specimen was examined using a SEM (Hitachi, S-3400N); the acceleration tension was 5.0 kV[30, 31].

R. fredii and *B. japonicum* bacteria were collected by centrifugation at 8000 rpm. Then the bacteria that were fixed for 20 min in the 2% glutaraldehyde in 0.1M PBS were washed with distilled water(×6). Then the bacteria were stained with 2% uranyl acetate for 5 min and washed with distilled water(×3). The stained bacteria were exposed to osmium tetraoxide for 5 min and the excess osmium tetraoxide was then discarded. The dehydration of the exposed bacteria to osmium tetraoxide was conducted with an acetone series (50%, 70%, 90%, and 100%, respectively) 5 min. Polymerization was done using pure epoxy resin in the embedding oven at 75°C/2 h and 90°C/2 h, after the bacteria were infiltrated by mixture of acetone and epoxy resin (1:1) for 15 min. The blocks were trimmed and cut to 90-nm, ultra-thin sections and mounted on 200-mesh, thin-bar copper grids (Agar). The specimens then were stained with Reynold's stain for 1 min. Each specimen was examined using a transmission electron microscope (TEM) (Hitachi, H-600) at an accelerating voltage of 100 kV. The length and width of the bacteria were measured by TEM, and an SPSS significance test was performed[32, 33].

RESULTS

Effect of plant growth regulator on bacterial population of Rhizobial

Table 2 shows the effects of plant growth regulator GA_3 and PIX on bacterial populations of *Rhizobial*. In this study almost all changes in bacterial population of *Rhizobial* were significant ($P < 0.05$) for inoculation treatments over control. Most bacterial populations of *R. fredii* and *B. japonicum* were increased by adding GA_3 in different concentrations to YMA solid medium. The significant increase in bacterial populations of *R. fredii* and *B. japonicum* reached a very significant level ($P < 0.01$) using Duncan's multiple range test while the GA_3 volume was 10^{-3} v/v over the control. This demonstrates that GA_3 applied at 10^{-3} v/v in the medium on bacterial populations of *R. fredii* and *B. japonicum* was the most effective. However, it was the opposite case for PIX. In this experiment, PIX applied at 10^{-1} to 10^{-6} v/v lowered the bacterial population of *R. fredii* and *B. japonicum*. The significant decrease in bacterial populations of *R. fredii* and *B. japonicum* reached a very significant level ($P < 0.01$) when the PIX volume was 10^{-2} v/v over the control. Figures 1 and 2 show the results.

Effect of plant growth regulator on the cell growth of Rhizobial

Figure 3 shows the cell growth of both *R. fredii* and *B. japonicum* increased in GA_3 solutions over control in concentrations ranging from 10^{-3} to 10^{-6} v/v. The absorbance reached 2.32 and 2.15, respectively, at 600 nm with the 10^{-3} v/v GA_3 of *R. fredii* and *B. japonicum* culture at 96 h. The absorbance was higher than for other treatments. However, the absorbance at 600 nm was very low when the concentration of GA_3 was 10^{-1} and 10^{-2} (Fig. 3 A, C). The situation was the same for bacterial populations of *R. fredii* and *B. japonicum* as we indicated previously (Fig. 1, 2). It revealed that the optimum concentration of GA_3 could induce an increase in the amount of *Rhizobial*. Figure 3 D shows that the cell growth of *B. japonicum* was reduced to varying degrees for all the concentrations of PIX over the control. The cells of *B. japonicum* could not grow in the PIX concentration of 10^{-1} in the medium; the curve shows no apparent change. The growth rate of *B. japonicum* was lower than for other treatments. The absorbance at 600 nm reached 1.57, which was the lowest value compared to other treatments when the concentration of PIX was 10^{-2} at 96 h of *B. japonicum* culture. With these results, we chose the concentration of GA_3 and PIX at 10^{-3} v/v and 10^{-2} v/v, respectively, as the optimum concentration for the following experiment. It is interesting to note that the cell growth of *B. japonicum* did not change significantly with the concentration of PIX except in the case of 10^{-1} over the control (Fig. 3 B). Explaining such differences could prove an interesting subject for further research.

Effect of plant growth regulator on pH of Rhizobial

Lochhead recorded that the optimum pH of the growth of *Rhizobial* was between 6 and 7[34], and this was confirmed by others[35]. As shown in

Figs. 4 A and C, within 96 h, the pH value was under 6 when the concentration of GA_3 was 10^{-1} and 10^{-2} v/v. Inhibition of *R.* strains is evident under pH 6, as shown in Fig. 3 A, C. However, the pH value of *R. fredii* and *B. japonicum* under 10^{-3} v/v of GA_3 stabilized between 6 and 7, and the rate of decrease in pH value was faster than other treatments including control. The pH value of *R. fredii* and *B. japonicum* under 10^{-3} v/v of GA_3 reached the lowest point, 5.9 and 7, respectively, at 36 h, and then remained stable. This is one reason why the plant growth regulator GA_3 induced an increase in the amount of *Rhizobial* strains. As Figs. 4 B and D indicate, within 96 h, the pH value was consistently above 7 when the concentration of PIX was 10^{-1} v/v. Inhibition of *Rhizobial* strains is evident above pH 7 as shown in Figs. 3 B and D. The pH value of *R. fredii* and *B. japonicum* increased to varying degrees for other treatments of different concentrations of PIX over control

Table 1. Primers developed in this study

Primer name	*Primer sequence* (5'-3')	*Fragment length*
*nod*A *F*	CGCAGCGGAATCTACGAG	226 bp
*nod*A *R*	GGTCGATTACGGCCTTAGGT	226 bp
*nod*D *F*	ATCTGTTGGTGGCGGAACT	242 bp
*nod*D *R*	CGGCAGGTTGAGATAGACATC	242 bp
338 F	CCTACGGGAGGCAGCAG	180 bp
518 R	ATTACCGCGGCTGCTGG	180 bp

Table 2. Effects of plant growth regulator GA_3 and PIX in different concentrations on average bacterial population of *R. fredii* and *B. japonicum*

Concentration of plant growth regulator (v/v)	Rhizobial- plant growth regulator			
	R. fredii -GA_3 (10^5 CFU·ml^{-1})	*R. fredii* -PIX (10^5 CFU·ml^{-1})	*B. japonicum* -GA_3(10^5 CFU·ml^{-1})	*B. japonicum* -PIX (10^5 CFU·ml^{-1})
0(CK)	13.0±0.2 dBCD	13.0±0.2 cB	12.6±0.1 bB	12.6±0.1 cC
10^{-1}	0 aA	0.5±0.4 aA	0 aA	0 aA
10^{-2}	3.0±0.4 bA	2.6±0.3 aA	0 aA	9.3±1.4 bB
10^{-3}	15.3±0.1 dD	9.5±0.3 bB	18.1±1.1 cC	10.9±1.1 bcBC
10^{-4}	13.5±0.9 dCD	11.0±1.8 bcB	14.1±0.4 bB	10.1±0.9 bBC
10^{-5}	9.9±0.3 cBC	10.8±1.0 bcB	13.5±1.5 bB	11.3±1.2 bcBC
10^{-6}	9.2±2.8 cB	12.9±1.6 cB	13.6±1.4 bB	12.4±0.1 cBC

Values followed by a different lower- or uppercase letter within each column are significantly different at 0.05 and 0.01 probability levels, respectively, using Duncan's multiple range test

Table 3. Effects of different concentrations of plant growth regulator GA_3 and PIX on length and width of *R. fredii* and *B. japonicum*

Treatment of plant *growth regulator*	Length or width-Rhizobial			
	L- *R.. fredii* (10^3 nm)	W- *R. fredii* (10^3 nm)	L- *B. japonicum* (10^3 nm)	W- *B. japonicum* (10^3 nm)
CK	2.79±0.15 bB	0.68±0.13 aA	2.25±0.26 bB	0.82±0.05 bA
10^{-3} v/v GA_3	1.93±0.35 aA	1.24±0.13 cC	2.84±0.17 cC	0.72±0.08 aA
10^{-2} v/v PIX	3.02±0.41 bB	1.01±0.12 bB	1.61±0.19 aA	0.83±0.06 bA

Values followed by a different lower- or uppercase letter within each column are significantly different at 0.05 and 0.01 probability levels respectively, using Duncan's multiple range test. L = length of *Rhizobial* cell. W = width of *Rhizobial* cell.

Fig. 1. Partial images showing the effect of the plant growth regulator GA_3 and PIX on the bacterial population of *R. fredii*

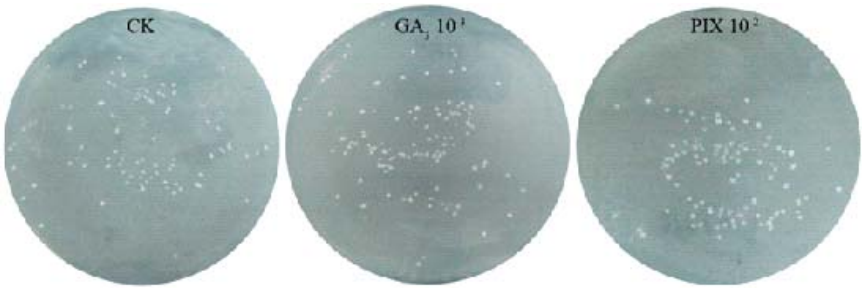

Fig. 2. Partial images showing the effect of the plant growth regulator GA_3 and PIX on the bacterial population of *B. japonicum*

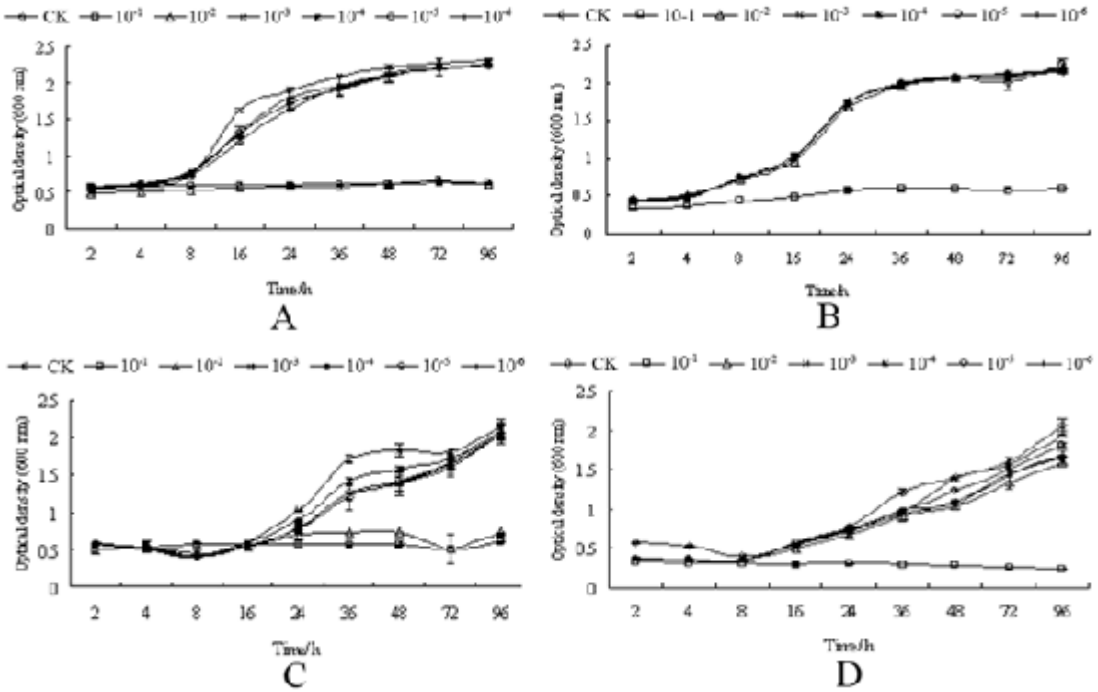

Fig. 3. Effect of plant growth regulator on *Rhizobial* cell growth. **A.** Effect of different concentrations of GA_3 on *R. fredii* cell growth. **B.** Effect of of different concentrations of PIX on *R. fredii* cell growth. **C.** Effect of different concentrations of GA_3 on *B. japonicum* cell growth. **D.** Effect of different concentrations of PIX on *B. japonicum* cell growth

throughout the 96 h growth period. During testing, the pH value of *R. fredii* and *B. japonicum* under 10^{-2} v/v of PIX reached the highest point, 7.2 and 7.8 at 16 h, then fell gradually, respectively. Even so, the pH value is still higher than for other treatments at the end of culture. This is considered

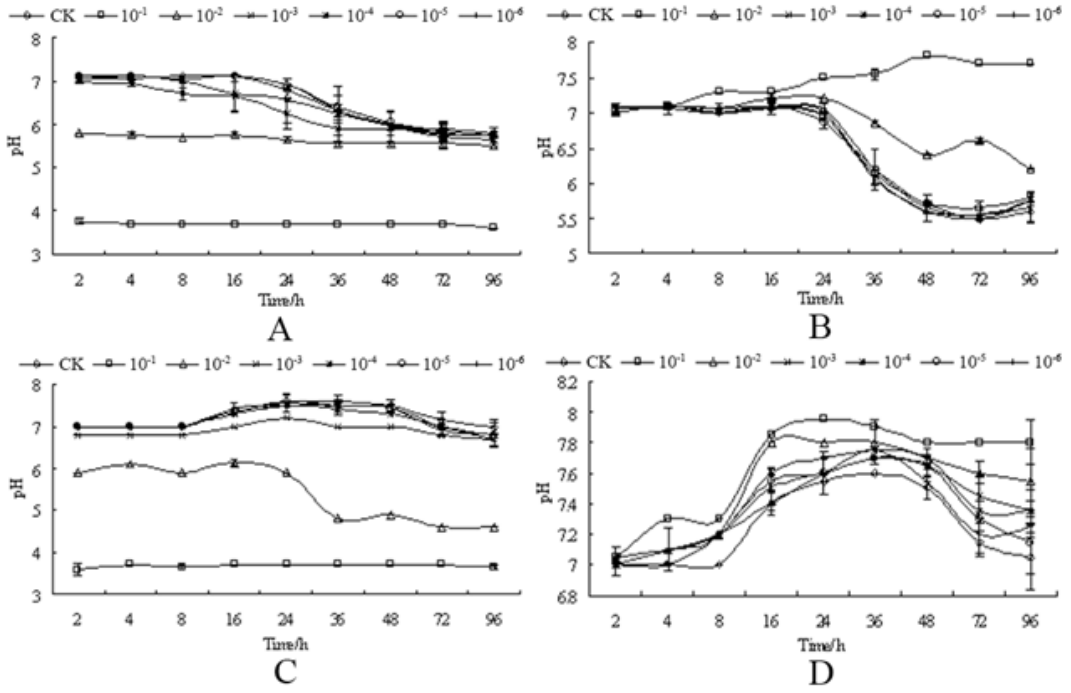

Fig. 4. Changes in pH value during the process of *Rhizobial* culturing in different concentration of plant growth regulator. A. Effect of different concentrations of GA_3 on changes in pH value of *R. fredii*. B. Effect of different concentrations of PIX on changes in pH value of *R. fredii*. C. Effect of different concentrations of GA_3 on changes in pH value of *B. japonicum*. D. Effect of different concentrations of PIX on changes in pH value of *B. japonicum*.

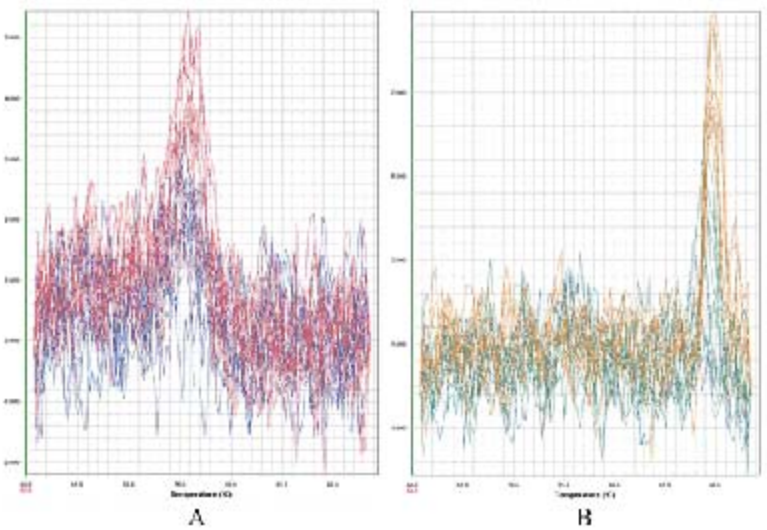

Fig. 5. Melting curve of quantitative-time PCR for *nod*A and *nod*D from *Rhizobial*. A. Melting curves for *nod*A amplicons from *R. fredii* (red curve) and *B. japonicum* (blue curve). B. Melting curves for *nod*D amplicons from *R. fredii* (green curve) and *B. japonicum* (orange curve).

one reason why plant growth regulator PIX inhibited growth of *Rhizobial* strains.

Effect of plant growth regulator on nodA and nodD expression of Rhizobial

As *nod* gene is involved in the development of nodule formation, we wanted to

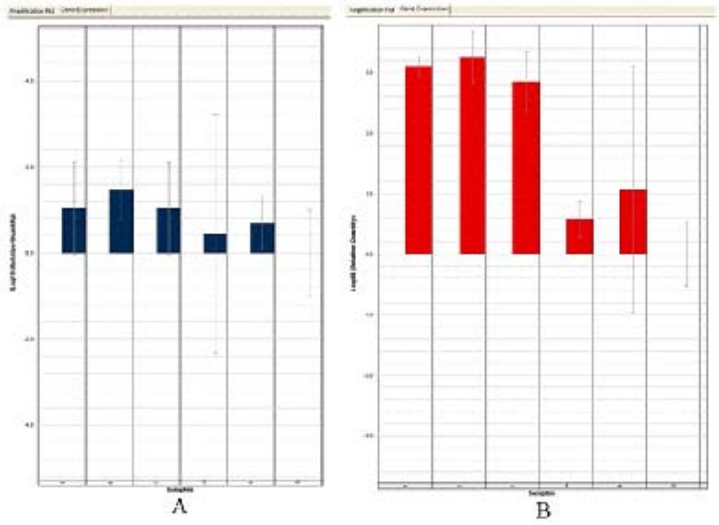

Fig. 6. Relative expression of quantitative-time PCR for *nod*A and *nod*D from *Rhizobial*. **A.** Relative expression of *nod*A amplicons from *R. fredii* (X axis-1, 2, 3) and *B. japonicum* (X axis-A, B, C). **B.** Relative expression of *nod*D amplicons from *R. fredii* (X axis-1, 2, 3) and *B. japonicum* (X axis-A, B, C). **X axis-1.** Relative expression of *R. fredii*. **X axis-2.** Relative expression of *R. fredii* with the GA_3 concentration of 10^{-3}. **X axis-3.** Relative expression of *R. fredii* with the PIX concentration of 10^{-2}. **X axis-A.** Relative expression of *B. japonicum*. **X axis-B.** Relative expression of *B. japonicum* with the GA_3 concentration of 10^{-3}. **X axis-C.** Relative expression of *B. japonicum* with the PIX concentration of 10^{-2}.

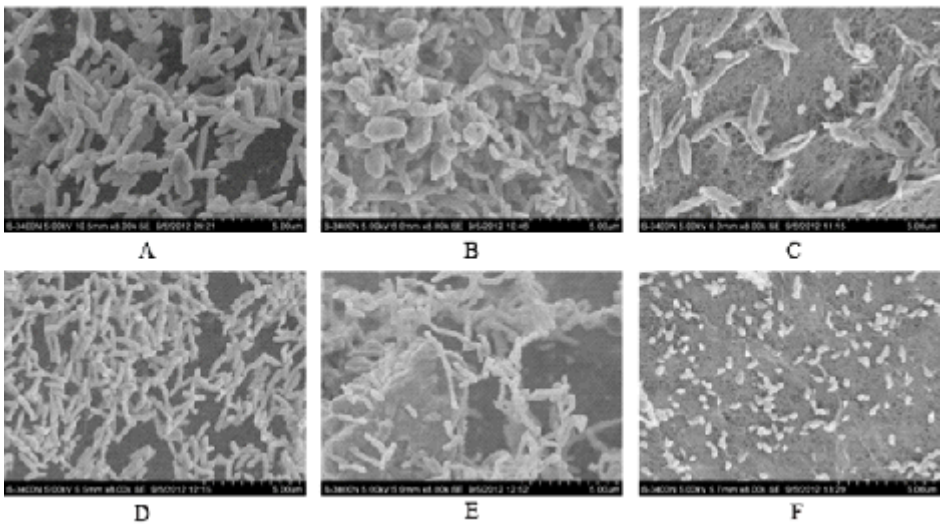

Fig. 7. Scanning electron micrographs of *Rhizobial* in different concentrations of plant growth regulator. **A.** Microstructure of *R. fredii*. **B.** Microstructure of *R. fredii* with the GA_3 concentration of 10^{-3} in the medium. **C.** Microstructure of *R. fredii* with the PIX concentration of 10^{-2} in the medium. **D.** Microstructure of *B. japonicum*. **E.** Microstructure of *B. japonicum* with the GA_3 concentration of 10^{-3} in the medium. **F.** Microstructure of *B. japonicum* with the PIX concentration of 10^{-2} in the medium.

measure the relative transcript abundance of *nod*A and *nod*D after GA_3 or PIX treatment in *R. fredii* and *B. japonicum*. To assay the selectivity on RNA extracted from *Rhizobial*, the presence of spurious amplification products was checked in real-time PCR by performing qualitative analysis of melting curves on the amplicons obtained from RNA extracted from *R. fredii* and *B. japonicum* under 10^{-3} v/v of GA_3 and 10^{-2} v/v of PIX. Only single melting main peaks with the same Tm (75.4°C for *nod*A, 89.5°C for *nod*D) were scored from all assays (Fig. 5), suggesting the absence of spurious amplification products from *R. fredii* and *B. japonicum* RNA.

The nodule formation of *Rhizobial* is believed to be regulated by PGRs. To search for PGRs with a similar regulated pattern on *nod* gene of *Rhizobial*, we analyzed the expression of the *nod*A and *nod*D genes at 96 hours after inoculation (the cell growth of both *R. fredii* and *B. japonicum* reach to the most thrive) with 10^{-3} v/v of GA_3 and 10^{-2} v/v of PIX respectively. And the histograms of the target gene expression were obtained by software SDS. From Fig. 6, the level of *nod*A and *nod*D expression of *R. fredii* and *B. japonicum* under 10^{-3} v/v of GA_3 were up-regulated, but not significantly. It is indicated that the *nod* gene expression can be induced by proper concentration of GA_3. Conversely, the situation of PIX at 10^{-2} v/v was down-regulated, thereinto, the suppressive effects of *nod*A and *nod*D expression of *R. fredii* was not significantly. But, *nod*A and *nod*D expression of *B. japonicum* under 10^{-2} v/v of PIX were almost not detected (Fig. 6 A-C, B-C). It is indicated that the *nod* gene expression can be inhibited by proper concentration of PIX.

Effect of plant growth regulator on microstructure of Rhizobial

To analyze plant growth regulator function in the microstructure of *Rhizobial* strains, 10^{-3} v/v of GA_3 and 10^{-2} v/v of PIX were inoculated into *R. fredii* and *B. japonicum*, respectively. Micrographs of *R. fredii* and *B. japonicum* observed by SEM and TEM showed distinctive features of the plant growth regulator–infected cells. Figures 7 A, B and 8 A, B show that most *R. fredii* cells under 10^{-3} v/v of GA_3 were round and the quantity of polyhydroxybutyrate granules[36] (Fig. 8, PHB) in *R. fredii* was induced to increase by GA_3 compared with the control. Results showed that the average length of *R. fredii* cells decreased from 2793 nm to 1928 nm and the width increased from 684 nm to 1240 nm by inoculating 10^{-3} v/v of GA_3, which reached a very significant level ($P < 0.01$) by Duncan's multiple range test (Table 3). The change in average width from 684 nm to 1007

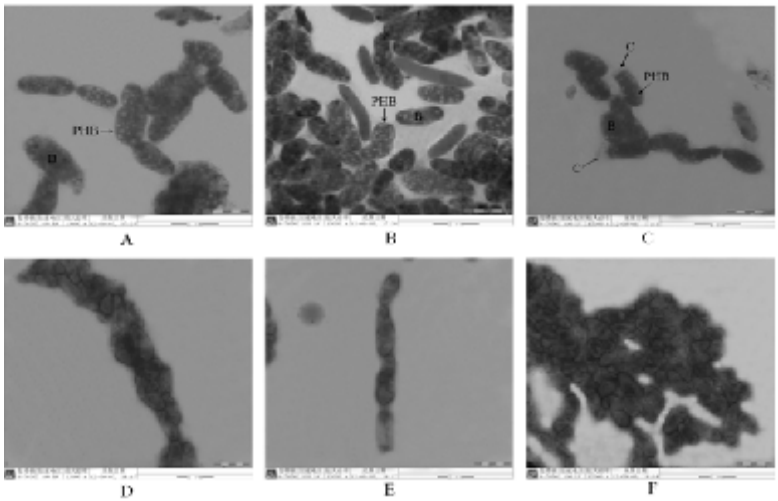

Fig. 8. Transmission electron micrographs of *Rhizobial* in different concentrations of plant growth regulator. A. Microstructure of *R. fredii*. B. Microstructure of *R. fredii* with the GA_3 concentration of 10^{-3} in the medium. C. Microstructure of *R. fredii* with the PIX concentration of 10^{-2} in the medium. D. Microstructure of *B. japonicum*. E. Microstructure of *B. japonicum* with the GA_3 concentration of 10^{-3} in the medium. F. Microstructure of *B. japonicum* with the PIX concentration of 10^{-2} in the medium. B = bacteroids. PHB = β-polyhydroxybutanic acid granule. C = capsule

nm in *R. fredii* cells by inoculating 10^{-2} v/v of PIX was very significant ($P < 0.01$). However, the increase in average length of *R. fredii* cells from 2793 nm to 3023 nm was less remarkable at 5% and 1% level of probability (Table 3). As Figs. 7 A, C show, both ends of *R. fredii* cells under 10^{-2} v/v of PIX were sharper than the control. That may be because a large number of *R. fredii* capsules were induced to produce on the cell surface by the plant growth regulator PIX, thereby altering the shape of *R. fredii* cells. This also explained the phenomenon that although the number of the *R. fredii* colony under 10^{-2} v/v of PIX was fewer, the absorbance at 600 nm did not changed markedly compared with the control (Fig. 8 A, C). In addition, the quantity of PHB in *R. fredii* was reduced by PIX, as shown in Figs. 8 A, C.

The effects of plant growth regulator on *B. japonicum* and *R. fredii* were different. As Figs. 7 D, E show, with 10^{-3} v/v of GA_3 the length of *B. japonicum* cells increased and the width decreased. As shown in Table 3, the change in length from 2246 nm to 2844 nm was very significant ($P < 0.01$) using Duncan's multiple range tests. The change in width from 815 nm to 719 nm was less remarkable at 1% level of probability (Figs. 8 D, E). However, the size of almost all *B. japonicum* cells with 10^{-2} v/v of PIX was quite small compared with the control (Figs. 7 D, F). As Figs. 8 D, F show, the change of length from 2246 nm to 1607 nm based on Table 3 was very significant at 5% and 1% level of probability but the change in width from 815 nm to 829 nm based on Table 3 was less remarkable at 5% and 1% level of probability.

DISCUSSION

Although plant growth regulator was applied intensively, comprehensive results of its biological impact on rhizobia–legume symbiosis were very limited. In our study, *R. fredii* and *B. japonicum* were selected as test strains due to their distinguishing physiological features of when grown on medium containing yeast extract and Mannitol, and within the subsequently nodulated plant hosts[37, 38]. In our study, the rate of growth and quantity of *Rhizobial* was directly affected by the type and concentration of plant growth regulators added to culture medium. These results were similar to those observed by Stearn et al. in which *R. japonicum* and *R. phaseoli* differed in their responses to PGR type (IAA, GA_3, and kinetin) and concentration[39]. Islas-Flores et al. also demonstrated that expression of RACK1 (PvRACK1) mRNA, responsible for nodule meristem initiation and *Rhizobial* nodule infection, was induced by PGRs (Auxins, Abscisic acid, Cytokinin, and Gibberellins)[40]. Our data suggest that relative expression of *nod*A and *nod*D genes of free-living cultures of *R. fredii* and *B. japonicum* under 10^{-3} v/v of GA_3 were inordinately up-regulated by Real-Time PCR Assay, compared with control. It is directly demonstrated that the quantity of *Rhizobial* were significantly induced following exposure to the *nod* gene of *Rhizobial* overexpression were induced by the suitable concentration of GA_3. While, PIX negatively regulate the expression of *nod* gene, which is associated with the decrease in the quantity of the pure cultured *Rhizobial*. Catriona et al. reported that the *nod*D viciae genes exhibited lower numbers when Zn was present, but could still be detected at appreciable levels in even the highest level of Zn contamination, whereas *nod*D trifolii genes were below detection limits in the highest level of Zn contamination[41]. Catherine et al. report that the pea early nodulin genes *PsENOD5* and *PsENOD12A* are induced during the interaction of pea roots and the endomycorrhizal fungus Gigaspora margarita[42]. Interestingly, the expression of *nod*D of free-living cultures B. japonicum. under 10^{-2} v/v PIX was not detected by RT-PCR assay, consequently, the expression of *nod*A of *B. japonicum* was inhabited (*nod*A expression regulated by *nod*[19, 20]). However, *B. japonicum* still grew in the culture medium. These results suggest that *nod* gene was not the only factor that affected the growth of *Rhizobial* in the culture medium.

Our results indicate that the quantity and growth rate of *Rhizobial* are both significantly induced following exposure to a GA_3 concentration of 10^{-3} (10 ppm), whereas lower and higher concentrations had either negative or inhibitory effects, respectively. These data confirm that presented in Elwan et al. in which 10 ppm also induced a significant increase in growth (final DW)[43], and in Fletcher et al. in which 25-1000 ppm GA_3 had no effect on the growth of *R. trifolii*[44]. Differences in nodulation responses in field-grown leguminous crops may therefore be related to

genotypic differences in endogenous Gibberellins production within these crops, further confounding data observed following foliar application of such compounds. PIX have been previously demonstrated to suppress vegetative growth[45-47]. However, our study exposed *Rhizobial* alone to PIX (at 10^{-2}), inducing a significant decrease and deceleration in the quantity, *nod* gene expression and growth-rate of *Rhizobial.* To our knowledge, this is the first report of the direct affect of PIX on free-living cultures of *Rhizobial*.

Among the many soil properties that influence the growth rate of *Rhizobial*, soil pH is of fundamental importance. Yang et al. demonstrated that very acidic or very alkaline environments are inhibitory to both nodulation and subsequent growth of the *Rhizobial*[48]. Yu et al. reported that the optimum pH for *Rhizobial* growth was between 6.0 and 7.0 and relatively few *Rhizobial* grew well at pH less than 5.0[49]. Morón et al. demonstrated that pH also had a role in inducing expression of *nod* genes, and on the structures of nodulation factors produced by *R. tropici* CIAT899[50]. In this study nodulation-related gene expression was greatest at pH 4.5 when compared with expression at a neutral pH. In our study, pH changed continuously during *Rhizobial*, likely in response to PGR type. The quantity and growth rate of *Rhizobial* increased and accelerated following addition of GA_3, which also resulted in a rapid decrease of pH. Conversely, when exposed to PIX the quantity and growth-rate of *Rhizobial* decreased and decelerated, subsequently increasing pH. Indole acetic acid (IAA) producing *Rhizobial* sp. bacterium isolated from the rhizosphere by others[51,52] revealed that these species sp. produce relatively high amounts of IAA during growth in basal medium supplemented with L-tryptophan. Therefore, it may be possible that differences in pH observed in this, and other studies may be a result of either stimulation or suppression of IAA production and secretion. It will likely be of value to explore these phenomena in greater detail in subsequent studies.

Our study found that the microstructure, including the length and width of *Rhizobial* cells, was changed significantly by plant growth regulator. Sauret-Güeto et al. indicated that the phytohormone gibberellin promotes plant growth by stimulating cellular expansion and the GA-induced increase in hypocotyl cell elongation rate is not dependent upon the maintenance of transverse orientation of the outer tangential wall MTs[53]. Lovett and Campbell reported that the growth retardants cycocel and mepiquat chloride inhibit cell elongation, resulting in reduced leaf expansion without any anatomical changes in the leaves, thereby increasing leaf thickness. Most scholars have drawn similar conclusions[54]. In addition, it was of interest to us that based on the transmission electron micrographs the amount of PHB in *R. fredii* was changed by plant growth regulator. There have been no such reports to date although Choi et al. reported elevated levels of NADPH significantly enhanced PHB accumulation by using *Ralstonia eutropha*[55]. The effect of different plant growth regulators on *Rhizobial* merits further study.

ACKNOWLEDGMENTS

We thank the National Key Technology Support Program (2012BAD20B04) and New Century Excellent Talents of the Heilongjiang Provincial University Program (NCET-06-002) of China for funding this project. We thank Weidong Wang and Yang Lin for their expert technical help. We also thank Naijie Feng, Tao Liu and Hui Wang for advice and assistance.

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