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EFFECT OF PHOSPHATE SOLUBILIZING RHIZOBIUM AND NITROGEN FIXING BACTERIA ON GROWTH OF ALFALFA SEEDLINGS UNDER P AND N DEFICIENT CONDITIONS

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Abstract

A sand culture experiment was conducted to evaluate the effect of nitrogen-fixing and phosphate-solubilizing *Klebsiella sp.* RSN219 and *Rhizobium meliloti* LW135 on seedlings of alfalfa (*Medicago sativa* L.) on the condition of nitrogen free and tricalcium phosphate only. Surface sterilized Alfalfa seeds were sown in polyvinyl plastics pots filled with clear sand. Insoluble phosphate was applied as tricalcium phosphate before sowing. Alfalfa seeds were inoculated with *Rhizobium* LW135 or *Klebsiella* RSN219. Results showed that the seedling was significantly promoted when seeds were inoculating with RSN219 or LW135. All the growth attributes of seedlings were significantly better than CK1 (1/4th Modified Hoagland's nutrient solution was offered, nitrogen and phosphorus free) and CK2 (1/4th strength Hoagland's solution was offered). Inoculation with LW135 was found better in plant growth promoting capabilities than RSN 219. Both of the 2 strains have good growth promoting capability, and effective in reducing growth inhibition of alfalfa seedlings under condition of nitrogen- and phosphorus-deficient.

Introduction

Alfalfa (*Medicago sativa* L.) was widely planted in arid and semi-arid areas (Safarnejad, 2008), and over 32 million hectares in the world (Mohamed *et al*., 2009; Xie *et al*., 2013) because of its forage, nutritive and high biomass producing values. The use of chemical fertilizers can temporarily relieve the deficiency of fertility, while the long-term use increased not only the cost of agricultural production but also the deterioration of soil and the selective accumulation of some chemical elements which is harmful to the environment (Mukhtar *et al*., 2013).

Since the discovery of nitrogen-fixing function in legume plants in 1886, the *Rhizobium*, microorganisms are successfully cultured and widely used to inoculate ehe legume seed to improve their n-fixing capability. Currently, global available nitrogen fixed through nitrogen-fixation bacteria (Include *Rhizobium*) for one year has exceeded to 2×10^9 Kg (Sheng & Jing, 2003).

As a main element in plant growth, P is the major limited nutrient for agricultural crops. In acid or alkaline soils, P always tightly binds with aluminum, iron, calcium and magnesium to form insoluble compounds or sparingly soluble phosphates, and unavailable for plant uptake (Holford, 1997; José *et al*., 2002). Phosphate solubilizing microorganisms could produce some organic acids and enzymes that transformed sparingly soluble phosphates into substances that could be easily assimilated by plants and therefore ameliorate the phosphorus supplying situation in soil (Rodríguez & Fraga, 1999; Babana *et al*., 2013). The application of nitrogen-fixing and phosphate solubilizing microorganism could decrease the use of chemical fertilizer and the pollution of underground water, renovate the ecological environment of soil and increase the yield and quality of plants (Lee & Bressan, 2005; Chen *et al*., 2006).

Nitrogen-fixing and phosphate solubilizing *Klebsiella* RSN219 and phosphate solubilizing *Rhizobium* LW135 were used as tested strains in the study, and perennial forage grass alfalfa which was widely used in northern China was chosen as tested material. The effects of RSN219 and LW135 bacterial solutions on the growth of alfalfa were studied under the condition of nitrogen free and poor phosphorus to provide basis and fertilizer efficiency parameters for the application of the two strains and the development of nitrogen-fixing and phosphate-solubilizing bacterial fertilizer.

Materials and Methods

Strains: The nitrogen-fixing and phosphate-solubilizing strain (*Klebsiella* sp.) RSN219 had been identified as *Klebsiella pneumoniae* by Chinese scientists [General Microbiological Culture Collection Center (CGMCC)] and according to biochemical tests 1.5-kbp sequence of the 16S rRNA gene region was fully found in it (Li *et al*., 2011). Phosphate-solubilizing *Rhizobium meliloti* LW135 (Li *et al*., 2009a) isolated from root nodules of alfalfa (*Medicago sativa*. L) plant, nodules were collected in mid-June 2008 at Lanzhou forage station of Gansu Agricultural University Gansu Province, People's Republic of China.

Both RSN219 and LW135 were maintained in yeast mannitol agar (YMA) solid medium (Zarrin *et al*., 2006) at 4^{o}C until needed for experiments in key laboratory of Grassland ecosystem of Ministry of Education, College of Grassland Science, Gansu Agricultural University.

Plant materials: Long Dong (*Medicago sativa. cv.* Long Dong) seeds were provided by College of Grassland Science, Gansu Agricultural University; while Golden

Empress (*Medicago sativa. cv.* Golden Empress) seeds were obtained from Clover Seed and Turf Co. (Clover Seed & Turf Co. Beijing. PR China). When the experiment started, seeds had stored for one year at room temperature, and the purity of seeds was 97%. Seeds were surface sterilized in aqueous solution of mercuric chloride (0.1%) for 2 min, and thoroughly rinsing with deionized water for 5 to 8 times (Zarrin *et al*., 2006).

Preparation of bacterial suspension: Agar slant cultures of RSN219 and LW135 that preserved at 4 ^{o}C for activation, cells of two strains were inoculated in Yeast mannitol liquid medium (YEM) (Li & Alexander, 1988) and cultured at 28^{o}C for 18 hours as rotation- shaken culture (120 rpm) until the absorbance value at 600nm reached 0.5. The solutions were centrifuged at 3,500×g for 10 min (Centrifuge Hermle, 2323 K, Germany), and then the cells were washed down with aseptic water and were shaken up and broken up. By now, bacterial suspensions with the OD value at 0.5 were prepared. Water the treatment on the 6th day of germination with 4 replicates for each treatment was applied.

Nitrogen and phosphorus free Hoagland's solution: Based on Hoagland's solution (Hoagland & Arnon, 1938), KNO_3 was changed into KCl, 582 mg liter^{-1}, without $(NH_4)_3PO_4$. The pH value was adjusted to 7.0±0.1.

Experimental design and treatments: The experiment was set up in April of 2010 at College of Grassland Science, Gansu agricultural university. Clean river sand was used as growing media after soaked in 4 mol L^{-1} HCl for 48h, rinsed for 6 times with distilled water and finally dried in an oven at 150^{o}C and sterilized for another 6h then 800 cm^{3} dried river sand was put into 1000ml plastic pot (diameter: 12cm), and tricalcium phosphate was added at the ratio of 20g Kg^{-1}. Twenty five grains each of Long Dong and Golden Empress were selected and evenly scattered onto the surface of sand and covered with 200g sand. Pots irrigated with 1/4th Hoagland's solution only were used as CK2; All treatments except CK2 were irrigated with 1/4th Hoagland's solution (Hoagland & Arnon, 1938) without phosphate and nitrogen to the maximum water-holding capacity after sowing (Khan *et al*., 2009), and 50ml RSN219 or LW135 suspension solution or 1/4th nitrogen and phosphorus free Hoagland's solution (CK1) was added when 30% of the water in pots was evaporated, and watered again on the 7th day after germination (CK2 was identically treated with 1/4th Hoagland's solution). Each treatment consisted of 5 replicates.

Moisture content of each pot was weighted every 3 days during the whole processing procedure, and was maintained at 70% of the maximum water-holding capacity with aseptic water. Moisture content was replenished to its maximum water-holding capacity with 1/4th (nitrogen and phosphorus free) and full (for CK2 only) Hoagland's solution every 10 days. All the indices were examined 50 DAT (day after treatment).

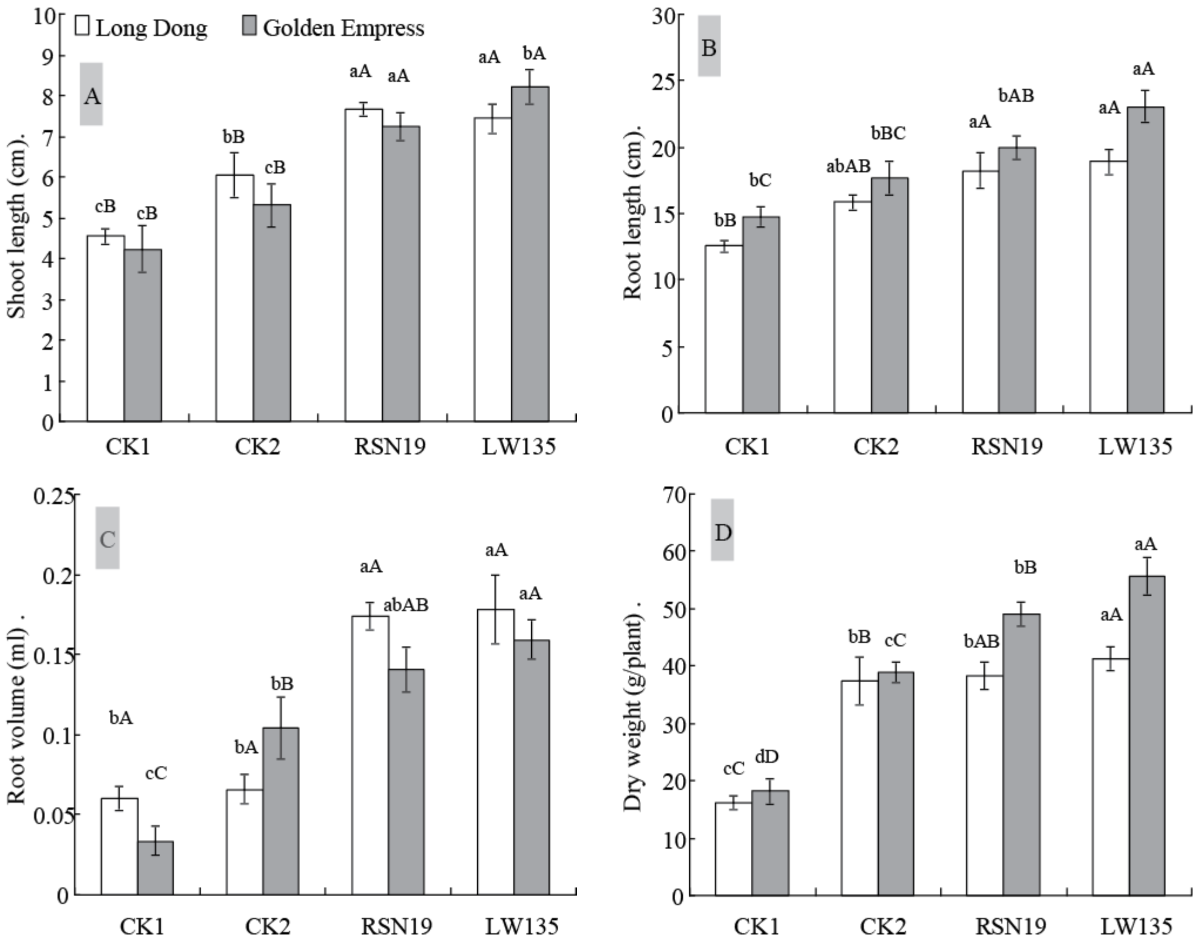
Harvesting schedule and test items: Five replicate seedlings (randomly chosen) of each treatment were used to be examined at 50 DAT. Shoot root lengths were measured in cm with ruler. Root volume of seedlings was tested by the water displacement method using 5 ml cylinder with minimum scale of 0.05 ml. The seedlings were oven dried at 75°C for 72h till constant weight and dry weight of seedlings in each treatment were recorded. Data of fresh weight were calculated on per plant bases. Nodules were separated from seedling roots and counted and their diameters were measured. *Before harvest*ing leaf area, photosynthetic activity, were measured in the second leaf from top of each plant. Leaf area of five replicate seedling chosen randomly in each treatment measured with a CI-203 Leaf Area Meter (CI-203, CID, Inc, USA). The chlorophyll contents of plants were extracted by homogenizing 0.1g fresh leaves with 10ml of 80% acetone. The mixture was centrifuged at 5000 rpm for 10 minutes, and was determined according to the method of Shan *et al*., (2008). Absorption measurements were recorded at wavelength of 445, 645 and 663nm using a Spectrophotometer (S2000, WPA Co., UK), and chlorophyll contents were calculated based on equations described by Arnon (1949).

The quantum yield of Photosystem II photochemistry (*Fv/Fm*) and the potential activity of Photosystem II photochemistry (*Fv/Fo*) were indirectly *measured* by using a fluorimeter (FMS-2, Hansatech Instruments Ltd, UK).

Statistical analysis: Data regarding all of parameters were subjected to analysis of variance and differences among the treatments were compared by least significant difference (LSD) test (Siddiqi *et al*., 2010).

Results

Effect of phosphate solubilizing bacteria on seedling growth: Of the 2 isolates have the ability of phosphate solubilizing and nitrogen-fixing; both of them significantly increased seedlings growth at 50 DAT (Fig. 1). After the inoculation of RSN219 and LW135, the shoot lengths of Golden Empress and Long Dong seedlings were increased by at least 63.8% over CK1 (p 0.05), and was increased by at least 23.5% over CK2 (p 0.01), respectively (Fig. 1A). The root lengths of Golden Empress and Long Dong seedlings were increased by at least 35.2% over CK1 (p 0.05), and was increased by at least 13.2% over CK2 (p 0.01), respectively (Fig. 1B). Root volume of Long Dong and Golden Empress seedlings inoculated with phosphate-solubilizing bacteria were enhanced 131.6% over CK1, and was increased 59.4% over CK2 (p 0.01) (Fig. 1C). Biomass of alfalfa seedlings inoculated with phosphate-solubilizing bacteria were increased 136.3% over CK1 (Fig. 1D), and P uptake percentage of inoculated seedlings was increased 9.62% over CK1 (Fig. 1E).



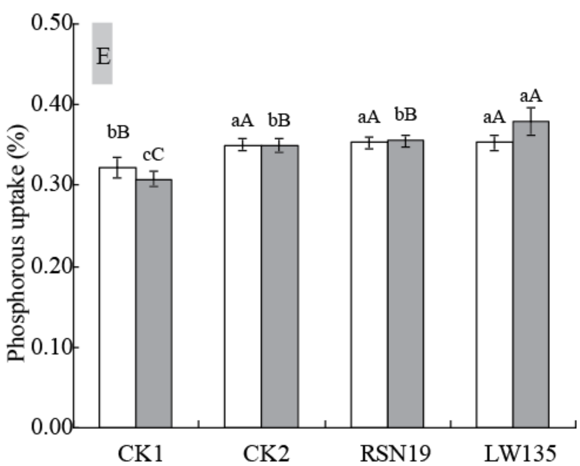


Fig. 1. Effect of 1/4th Hoagland's solution, *Klebsiella* RSN219 and *Rhizobium meliloti* LW135 application on shoot length (A), root length (B), root volume (C), dry mass (D), and Phosphorus uptake (E) of alfalfa seedling on conditions of nitrogen free and tricalcium phosphate only (without available phosphorus). Also shows shoot and root lengths, root volume and biomass of seedlings were *promoted more* effectively by LW135 *than* RSN219. Biomass and root length of Golden Empress seedlings were promoted by LW135 or RSN219 more effectively than Long Dong.

Application of promoted leaf area and number of leaves per plant in alfalfa seedling (Fig. 2A). In Long Dong and Golden Empress seedlings, phosphate *Rhizobium meliloti* inoculation significantly enhanced number of leaves, leaf area by 26.6% and 123% over CK1, respectively (p 0.01). The promoting effect of RSN219 on number of leaves of Long Dong was more evident than those in Golden Empress.

Effect of phosphate-solubilizing bacteria inoculation on photosynthetic characteristics: Seedlings inoculated by LW135 or RSN219 or from CK2 maintained nearly maximum quantum efficiency of PS photochemistry (*Fv/Fm*) (Fig. 3A) and PSII activity (*Fv/Fo*) (Fig. 3B). *Values* of *Fv/Fm* and *Fv/Fo in* inoculated *seedlings significantly higher than* those in CK1 (p 0.01). After inoculation of *Klebsiella* RSN219 and *Rhizobium* LW135, chlorophyll contents of seedling leaves were not significantly different between CK2, but significantly higher than CK1 (p 0.05).

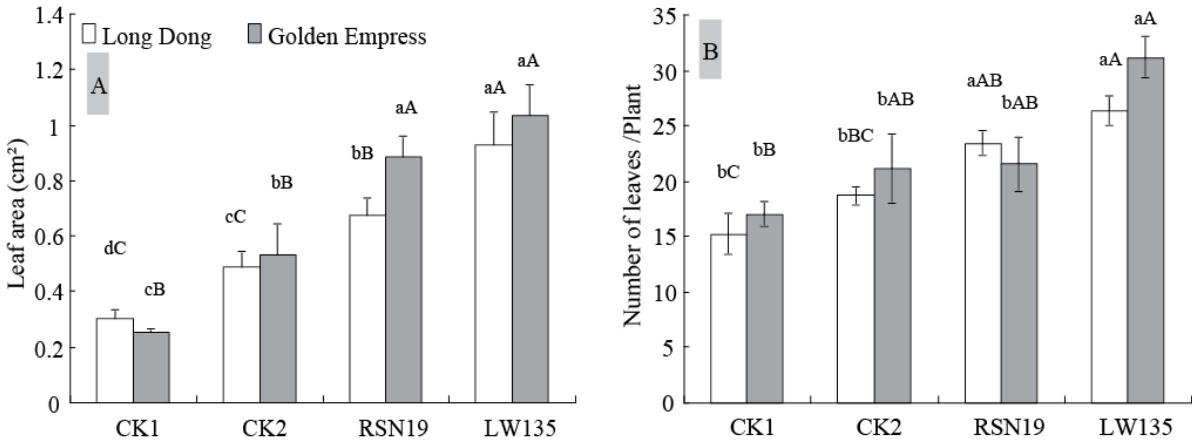


Fig. 2. Influence of 1/4th Hoagland's solution, *Klebsiella* sp. RSN219 and *Rhizobium meliloti* LW135 application on leaf area (A) and number of leaves (B) in alfalfa seedling on conditions of nitrogen free and tricalcium phosphate only (without available phosphorus).

Effect of phosphate-solubilizing bacteria inoculation on nodulation and seedling survival rate: Nodule diameters, number of nodule and survival rate of alfalfa seedlings were remarkably increased after the inoculation of RSN219 and LW135 (Table 1). The number of seedling (Long Dong & Golden Empress) nodules inoculated with LW135 was at least 5, 9 and 4 times higher than CK1, CK2 and those inoculated with RSN219, respectively. It is manifested that LW135 inducted the formation of nodules remarkably and the diameter of nodules enlarged after the inoculation. Although compared with CK1 and CK2, the number and diameter of nodules in RSN219 inoculated was increased, but it was still incomparable with those inoculated with LW135.

Table 1. Effect of *Klebsiella* RSN219 and *Rhizobium* LW135 on nodule diameters, nodule numbers and survival rate of alfalfa seedlings on conditions of nitrogen free and tricalcium phosphate only.

Treatment	Long dong			Golden empress		
	Survive rate (%)	Nodule No. (plant $^{-1}$)	Nodule diameter (mm)	Survive rate (%)	Nodule No. (plant $^{-1}$)	Nodule diameter (mm)
CK1	52.82	1.55	0.52	60.00	1.80	0.70
CK2	76.09	2.44	0.98	68.37	1.90	1.22
RSN219	84.50	3.16	1.37	88.56	7.10	1.68
LW135	88.76	22.12	2.54	86.33	28.66	2.61

CK1 was treated with 1/4th modified Hoagland's solution (nitrogen and phosphorus free) only; CK2 was treated with 1/4th Hoagland's solution; Values are means of five replicates; Percentage of survival seedlings in the recovery test was calculated as: survival seedlings/germinated seeds × 100

Discussion

The effects of RSN219 and LW135 on seedling growth: Two varieties of alfalfa seedlings were found obviously promoted after being inoculated with LW135 or RSN219. Survival rate of seedlings, shoot height, root length, root volume, leaf area, individual number of leaves per plant, biomass and P uptake percentage of the 2 *M. sativa* varieties were found remarkably increased than *control group (CK1)* (*p* 0.05). This shows that after the inoculation of phosphate solubilizing *Rhizobium* and *Klebsiella* bacteria the productivity and growth of alfalfa were efficiently promoted in soil that is deficient in nitrogen and available phosphorus. German *et al*., (2000) observed that associative nitrogen fixing bacteria and phosphate solubilizing bacteria play a positive role in promoting the increase of root length, plant size and root surface area, as well as the assimilation of water and nutrient, and help to strengthen the respiration in root (Sarig *et al*., 1992; Vedder *et al*., 1999); Vessey and Heisinger, (2001) also found that the accumulation of dry matter in pea was increased after the inoculation with a phosphate solubilizing fungus *Penicillium bilaii*.

Nitrogen fixation was divided into 4 types: symbiotic nitrogen fixation (Carroll, 2001), endogenetic nitrogen fixation, symbiotic nitrogen fixation and associative nitrogen fixation according to the habitat of nitrogen fixation and the relationship between nitrogen fixing microorganism and plants. Nitrogen fixation could only be carried out on anaerobic condition or nitrogenase could be inactivated rapidly. The bacterium "LW135" is a symbiotic nitrogen fixing type which combines with plant and forms an interiorly anaerobic structure the nodules, hemoglobin in nodules could make the bacteria to fixate nitrogen on proper anoxic and partial pressure conditions, and therefore provides nitrogen to plants. Bacteria RSN219 is belonged to a symbiotic nitrogen fixing catagory, who cannot fix nitrogen and form nodules on aerobic condition like *Rhizobium*, and additional energy is required to intensify respiration and to decrease oxygen concentration inside, or multiple polysaccharide should be secreted to form an anaerobic screen, therefore, the nitrogen fixation efficiency was generally not as effective as *Rhizobium*. The nitrogen fixation mechanism difference between strains might be one of the reasons that growth promoting effect of LW135 was better than RSN219, and it might also be the reason that phosphate solubilizing capabilities of the two strains were different. Phosphate solubilizing capability was believed as closely related with the kind of organic acid that the strain secreted. Zhang *et al*., (2013) also found that phosphate solubilizing capability of various bacteria were significantly different.

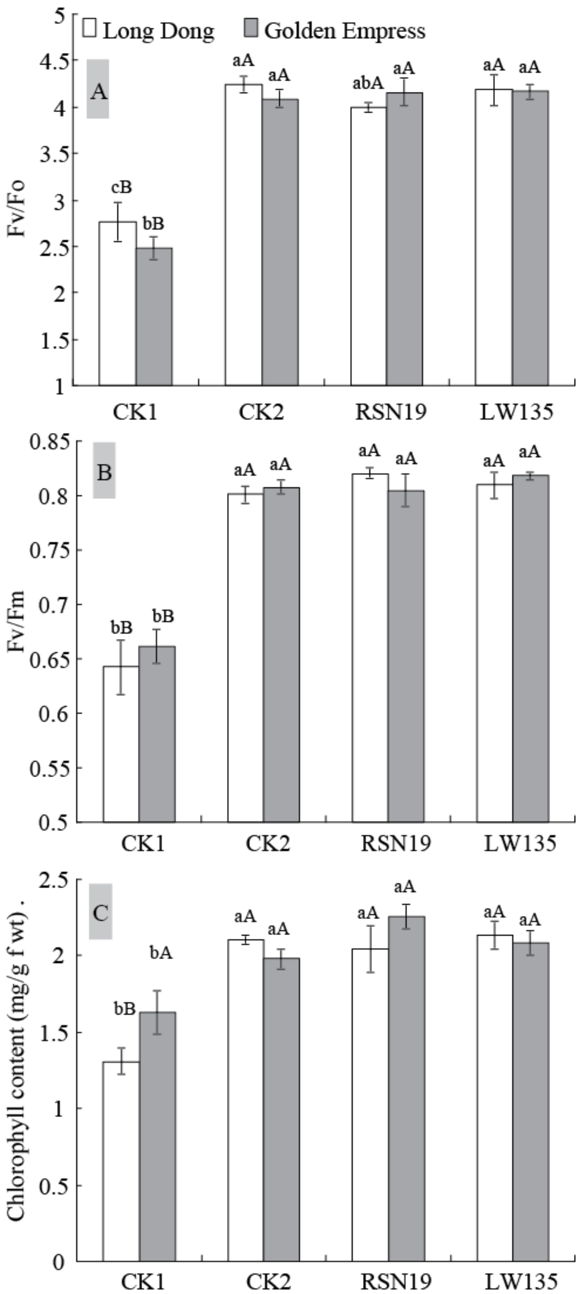


Fig. 3. Effect of 1/4th Hoagland's solution (CK2), *Klebsiella* RSN219 and *Rhizobium* LW135 application on PS photochemistry (A), PSII activity (B) and Chlorophyll contents (C) in alfalfa seedlings on conditions of nitrogen free and tricalcium phosphate only (without available phosphorus).

Attention should be noted that a few nodules were found within the treatments that did not inoculate Rhizobium. That is due to the endogenetic differences in *Rhizobia* (Li *et al*., 2009b; Zhang *et al*., 2009). Nodules could be inducted by the *Rhizobium* within seeds after the surface disinfection, while only a few nodules could be formed by this way which contributes little to growth promoting effect.

Growth promoting capability of LW135 on different alfalfa varieties was also various, this related highly close with symbiosis capability and the matched degree between *Rhizobium* and host plants. Nodules could only be formed after the mutual recognition between the host plant and the polysaccharide with distinct structure on the surface of strains, according to which nodulation gene was expressed, expression product was synthesized and morphological and psychological changes were taken place (Geurts *et al*., 2005). A single strain cannot provide all alfalfa varieties with the same inoculation outcome on the same culture condition. A strain that proved good to some variety might be poor to another variety (Kan & Chen, 1997).

Both of LW135 and RSN219 were found with nitrogen fixing and phosphate solubilizing activities, and obvious growth promoting effect was expressed. Rice & Lupwayi (2000) found that the cooperation of phosphate solubilizing bacteria and *Rhizobium* was good to increase the yield of crops. Combined inoculations with *N_2-fixing and P-solubilizing* bacteria were more effective than single inoculation (Erdal *et al*., 2008). The *Klebsiella* was the most abundant nitrogen-fixing bacteria (Shahida *et al*., 2004). As a symbiotic nitrogen fixing bacteria, *Klebsiella* RSN219 could not form nodules, but it was not limited by plant type, and therefore has good application potential.

It should be noted that the results obtained from our study based only on plants cultured in sand that grew indoor, the actual growth promoting effect in soil should be further studied by field experiment.

Conclusion

Inoculation of RSN219 and LW135 promote growth of alfalfa plants grown in 1/4 strength Hoagland's solution. Both the two strains increase the survival rate of alfalfa and the growth indices i.e., shoot and root lengths, individual leaf area, number of leaves per plant, root volume, biomass, phosphorus uptake percentage and maximum quantum efficiency of PS photochemistry (*Fv/Fm*) and PSII activity (*Fv/Fo*). Both the stains (LW135 and RSN219) were biotical nitrogen fixing and phosphate solubilizing and showed good growth promoting effects under nitrogen and available phosphorus free conditions. The stain LW135 showed better growth promoting effect than RSN219 in alfalfa seedling.

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