The Effects of Sap of *Nitraria* Plant on the Growth of Its Endogenous Nitrogen-fixing Microbes under Saline-Alkaline Stress

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Abstract—The endophytic N-fixing microbes *Klebsiella oxytoca* NHglj1, *Klebsiella oxytoca* NHglj2 and *Enterobacter asburiae* LMw107 of *Nitraria* were selected to explore the effect of *Nitraria schoberi* L. plant sap on salt and alkali resistance of its 3 endogenous N-fixing microbes strains in the Winogradsky's N-free medium under different salinity and alkalinity gradient. The results show that the NaCl tolerance of 3 strains is 0 ~ 4% and 3 strains can all survive in pH 6～13. After sap of *Nitraria* is added, the salt resistance of NHglj1 decrease, the colony diameter of K. oxytoca NHglj1 is 59% ~ 82% that of control, the change of alkali resistance of NHglj1 is similar to that of the salt resistance, the diameter of NHglj1 is 13% ~58% that of control (P<0.05). However, the salt resistance of NHglj2 and LMw107 increase, the diameter is larger than 2 %~ 45% that of the control. The alkali resistance of NHglj2 and LMw107 also increase in pH6~13. The results exhibit that *Nitraria* sap may partially improve salt-alkali resistance of parts of endogenous N-fixing microbes.

Keywords- Nitraria; Plant sap; Endogenous N-fixing microbes; Saline stress; pH

I. INTRODUCTION

Endogenous N-fixing microbes can utilize excess energy to fix nitrogen during the process of host plant tissue conduction and colonization, and macroscopically be regulated by plant regulating system [1], exhibits high N-fixation efficiency and promote host growth by secreting auxin, phosphate solubilizing, enhancing plant resistance and so on stress resistance mechanisms[2]. Endogenous N-fixing microbes have enormous potentiality to supply nitrogen and enhance environmental adaptability, especially under situation of harsh climate and lack of nitrogen [3], so it displays a good application prospect in ecological restoration [4-5]. Symbiotic relationship between endogenous N-fixing microbes and plant has been one of hot content of common concern in botany, microbiology, and ecology [6]. Environmental condition has an effect on invasion and colonization, N-fixation and promoting plant growth, enhancing host anti-adversity efficiency full play of endogenous N-fixing microbes [7-8], the key to playing the positive role of endogenous N-fixing microbes is if endogenous N-fixing microbes can effectively colonize and establish stability and harmony combination [9]. So there is an important significance of theory and practice to study symbiosis nitrogen fixation mechanism between plant and endogenous N-fixing microbes to understand adaptability of plant and microorganism, stress resistance, species diversity, and the role of ecological restoration under atrocious environment [10].

Nitraria schoberi. L is super-xerophytes, distributed in province of Inner Mongolia, Gansu, Xinjiang of China, and possesses the capabilities of drought tolerance, saline-alkaline, sand burial, and resistance to wind erosion [11]. Sand burial branches of N. *schoberi* can grew out new adventitious root and accumulate sand into dune in damp sand, and form fixed, semi-fixed shrubs, so it has very important ecological status in arid and semiarid areas. Nie, *et al.* reported that *Nitraria* get nitrogen by endogenous N-fixing microbes [12]. Herridge *et al.* confirm by 15N dilution method that some sugarcane obtained 60% nitrogen of total nitrogen content through endogenous N-fixing microbes [13].

The studies on *Nitraria* mainly involve vegetation succession, environmental adaptation, shifting sand fixation of its plant, in this research, we use the sap of *Nitraria* and endogenous N-fixing microbes as materials to explore the effects of plant sap on saline-alkali tolerance of plant endogenous N-fixing microbes, which reveal adaptability of *Nitraria* endogenous N-fixing microbes to rigor environment factor and the protection of *Nitraria* on strains. This research provides the materials and reference for exploring the symbiotic relationship

between *Nitraria* endogenous N-fixing microbes and host, and biological nitrogen fixation under desert environment.

II. Materials and Methods

A. Materials and Medium

Plant Materials and Habitat Survey of Plant Materials:

Minqin county of Gansu Province of China is one of typical oasises in desert, and bounded by Tenggeli desert and Badanjilin desert from three planes, sampling place belongs to temperate desert climate, and the annual evaporation is 2604.3mm. The majority landform type includes shrub coppice dunes and moving sand dunes [11].

Collection of Plant Materials: Wild *Nitraria schoberi* plants were collected at Xishawo area of Minqin county Gansu Province of China in August 2012, four semi-fixed sand dune plot with growing single community were randomly selected, five complete plants were collected at each plot. Fresh tissue samples and sandy soil were placed in chamber, and the original drying conditions were maintained as possible.

The Strains Tested: The strains indentified as *Klebsiella oxytoca* NHglj1, *Klebsiella oxytoca.* NHglj2, and *Enterobacter asburiae* LMw107 are separated and purified from root, stone, flesh of wild *Nitraria* separately.

Culture Media: Preparation of Winogradsky's N-free medium referred to Hara's method, N-free plates with 1.6% agar [14].

B. Test Method

The preparation of the diluents of *Nitraria* leaves: The fresh and healthy leaves of *Nitraria* were washed 3-4 times with distilled water, after surface moisture was sipped up with filter paper, 1.0 g leaf was accurately weighed by electronic balance, The fresh leaf was grinded with being added 10ml sterile distilled water in aseptic technique platform, after washing mortar with sterile water, leaf lapping liquid of *Nitraria* was filtered 2 times with 0.45um sterilized filter membrane, total 200ml sterile water was added in the whole process. Finally, diluents of surface sterile leaf were stored in refrigerator at 4℃.

Culture Medium: Basic culture with being cooled to about 40℃ was filled into Erlenmeyer flask (150ml), 49ml per bottle, according to the following test scheme to treatment setting.

a. Salt Resistance Test: salt concentration of basic culture was set at 0%, 1%, 2%, 3%, 4% and 5% through being added with NaCl.

b. Alkali Resistance Test: pH of basic culture was set at 5, 6, 7, 8, 9, 10, 11, 12 and 13, through being added with HCl, NaOH.

All above different gradient culture were sterilized at 121℃ by moist heat in 26min, after culture was taken out, leaf lapping liquid of *Nitraria* was added into each gradient culture medium and sterile saline of the same salt concentration and alkali concentration were served as control, at last they were marked as "Y" group with being added into sap of *Nitraria* and "N" control group separately.

Inoculated and Culture: In aseptic operation platform, 1ml sterile sap of *Nitraria* was separately added into all Y group gradient 150 ml Erlenmeyer flask with containing 49ml nitrogen-free culture and was shaken up, In addition 1ml sterile water was separately added into all "N" group, Erlenmeyer flask (150 ml) with containing 49ml N-free culture (50℃) and served as controls. 50 ml culture which was placed in Erlenmeyer flask (150 ml) was in poured into 9 cm Petri-dish. After culture was cooled solidification, three strains were streaked in N-free plates in all treatment, 4 replications per strain. Plates were cultured at 27℃ in a incubator at least 168 h until the colony diameter was measured.

C. Data Procession and Analysis

The data was presented as means±SE and differences of variables between treatments were compared using one way ANOVA followed by Dennett's test. The statistical software SPSS 16.0 was used.

III. Results And Analysis

A. The effects of sap of Nitraria on the colony diameter of endogenous N-fixing microbes under saline stress conditions

Fig .1 shows that the diameter of *K. oxytoca* NHglj1 exhibit significant differences (P<0.05 for all), after NHglj1 separated from the root of *Nitraria* is cultured for 168h under different treatment. Under culture conditions with being added leaf lapping liquid of *Nitraria* (followings replaced by "Y" group) and control group (equivalent sterile water replace leaf lapping diluents, followings replaced by "N" group), the NaCl tolerance range of NHglj1 was 0~4%, but NHglj1 can not survive under 5% NaCl. The results shows that the colony diameter decrease gradually with the increase of NaCl concentration under without being added sap of *Nitraria*, after being added sap of *Nitraria*, diameter increase firstly and then decrease gradually with the increase of NaCl concentration, and the diameter reaches the maximum value (3.11~3.00mm) in 1~2% NaCl. The diameter of Y group is respectively 18%, 40%, 41%, 38% and 31% of N group, The results exhibit that sap of *Nitraria* can significantly decrease salt resistance of NHglj1 of root(P<0.05)

Fig .2 shows that the diameter of *K. oxytoca* NHglj2 exhibit significant differences (P<0.05), after NHglj2 separated from the stone of *Nitraria* is cultured for 168h under different NaCl treatment. No matter with or without sap of *Nitraria*, the diameter of NHglj2 decrease with the increase of NaCl concentration in 0~5%, but NHglj2 can not grow under 5% NaCl. After being added sap of *Nitraria*, the diameter of Y group is respectively 1.04, 1.30, 1.18, 1.45, and 1.36 times of N group in 0~4% NaCl, the changes show significant differences (P<0.05) in 1~4% NaCl, the results suggest that sap of *Nitraria* can significantly increase salt resistance of NHglj2 of stone (P<0.05)

Fig .3 shows that the diameter of *E. asburiae* LMw107 exhibite significant differences (P<0.05), after LMw107 separated from the stone of *Nitraria* is cultured for 168h in solid culture under different NaCl concentration treatment. LMw107 is similar to NHglj2**,** no matter with or without sap of *Nitraria*, the diameter of LMw107 decrease with the increase of NaCl concentration in 0~5% NaCl, after being added sap of *Nitraria*, the diameter of

"Y" group is respectively 1.12, 1.05, 1.36 and 1.02 times of "N" group in 0～3% NaCl, 86% of N group under 4% NaCl, the changes in the diameter show significant differences (P<0.05) in 0~4% NaCl.

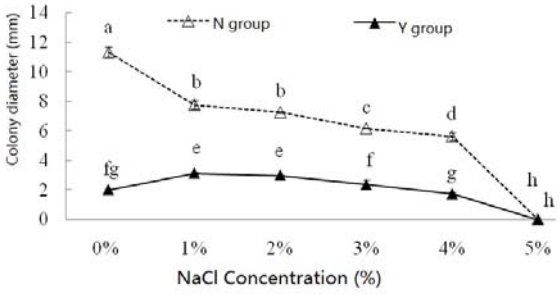

Figure 1. The effects of sap of *Nitraria* on the colony diameter of *K. oxytoca* NHglj1

* Note: the different small letters mean significant difference among the different treatments (P<0.05), results are means ± SD (n=4). same blew.

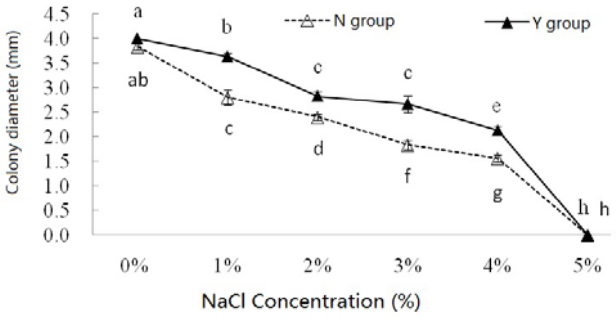

Figure 2. The effects of sap of *Nitraria* on the colony diameter of *K. oxytoca* NHglj2

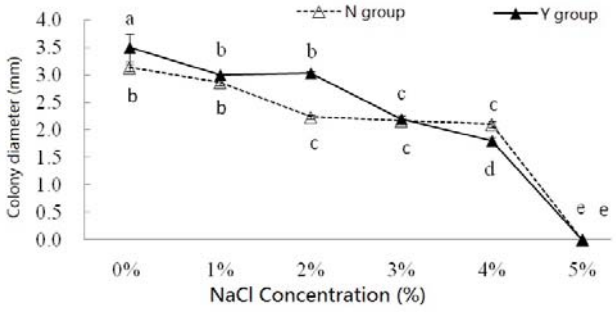

Figure 3. The effects of sap of *Nitraria* on the colony diameter of *E. asburiae* LMw107

B. The effects of sap of Nitraria on the colony diameter of endogenous N-fixing microbes under alkaline stress

Fig .4 shows that the diameter of *K. oxytoca* NHglj1 exhibite significant differences (P<0.05), after NHglj1 separated from the root of *Nitraria* is cultured in solid culture medium for 168h under different pH treatment. The diameter of Y and N group reach the maximum value in pH12. After being added sap of *Nitraria*, the diameter of Y group was respectively 35%, 34%, 35%, 35%, 34%, 58%, 51%, 13% of N group in pH 6~13% , the changes in the diameter show significant differences(P<0.05). The results suggest that sap of *Nitraria* can significantly decrease acid and alkali resistance of NHglj1 (P<0.05).

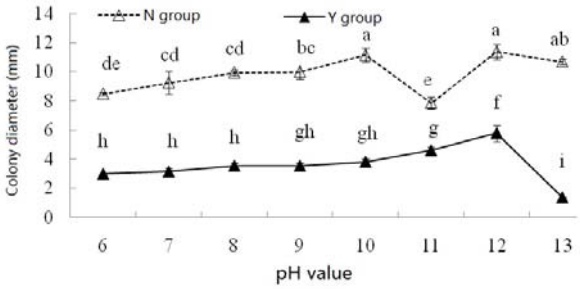

Figure 4. The effects of leaf diluents of *Nitraria* on the colony diameter of *K. oxytoca* NHglj1 under alkaline stress

Fig .5 shows that the diameter of *K. oxytoca* NHglj2 exhibit significant differences (P<0.05) in solid culture for 168h under different pH. Without the sap of *Nitraria*, the diameter of *K. oxytoca*NHglj2 reach the maximum value in pH 8; Maximum diameter performs in pH 7 under being added sap of *Nitraria*. The diameters of "Y" group are higher than those of "N" group in pH 6~13 (P<0.05). The results indicate that sap of *Nitraria* can significantly increase alkali resistance of NHglj2 (P<0.05) and improve colony forming speed of strains.

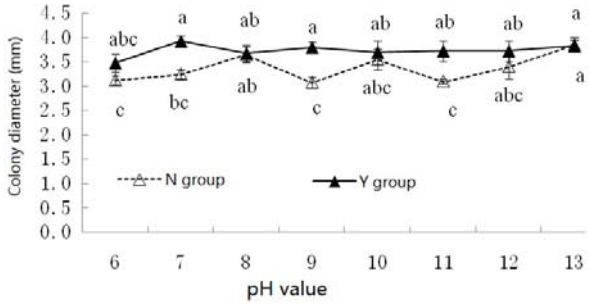

Figure 5. The effects of leaf diluents of *Nitraria* plant on the colony diameter of NHglj2 under alkaline stress

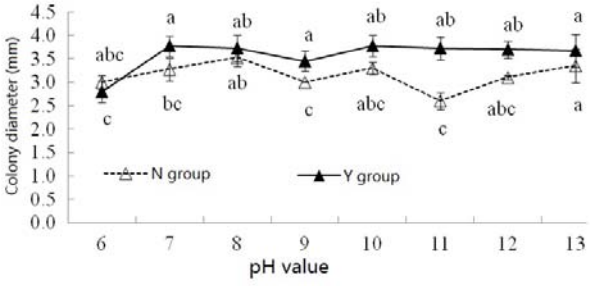

Figure 6. Effects of leaf sap of *Nitraria* plant on the colony diameter of *E. asburiae* LMw107 under alkaline stress

Fig .6 shows that the diameter of *E. asburiae* LMw107 exhibite significant differences (P<0.05), after LMw107 separated from the flesh of *Nitraria* is cultured in solid culture for 168h under different pH treatment. Under no sap of *Nitraria*, the diameter of LMw107 reach the

maximum value (3.53mm) in pH 8, maximum value （3.78mm）in pH 7 under being added sap of *Nitraria*. The diameters of Y group are higher than those of N group (respectively $P<0.05$) in pH 7~13. This result is similar to that of NHglj2, which exhibit that sap of *Nitraria* can significantly increase alkali resistance of LMw107 ($P<0.05$).

IV. Discussion

In this research, results show that 0.1g fresh leaf sap per liter leaf sap performs the best on improve colony growth. In order to guarantee plant tissue grinding fluid to sustain action in the growth period of N-fixing microbes, plant sap is immediately added into N-free medium before it solidifies, instead of smearing plant sap on the surface of the solidified medium, but the operation is been on, due to the temperature of medium comes to solidified is at about 50℃, which may be changed the composition or activities of some enzymes in plant sap.

The results show that the NaCl tolerance of 3 strains was 0~4%, 3 strains could not grow at 5% NaCl, which shows that alkali resistance of 3 strains was extremely strong.

Zhang report that the sap of alfalfa plant reduced more auxin, exogenous *Rhizobium* polysaccharide from *Sinorhizobium meliloti* 12531f, *Rhizobium meliloti* GNf and *Rhizobium meliloti* GN5, and also promote the growth and proliferation of rhizobia [15]. That uniform with manifestation of *K. oxytoca* NHglj2 and LMw107 in this research, but manifestation of NHglj1 is just contrary.

V. Conclusions

The results suggest that no matter whether sap of *Nitraria* is added or not, the NaCl tolerance of 3 strains was all in 0~4%, but 3 strains can not survive at 5% NaCl; with being added sap of *Nitraria*, the colony diameter of NHglj1 is significantly lower than that of control group ($P<0.05$), and is 18%~41% of control, However, the colony diameter of NHglj2 and LMw107 are 1.02~1.45 times than those of control group.

No matter sap of *Nitraria* is added or not, 3 endogenous strains separated from different tissues can grow over in pH 6~13 under N-free conditions, and has application potential of survival nitrogen fixation under alkaline stress. The colony diameter of NHglj1 is significantly lower than that of control group ($P<0.05$), and that means alkaline-resistance of NHglj1 is decreased when plant sap of *Nitraria* is added to. However, the colony diameter of NHglj2 and LMw107 are much greater than those of control group over in pH 6~13. These results indicate that the sap of *Nitraria* can improve the adaptation for parts of endogenous N-fixing microbes to acidic and alkaline environments. But hosts plant may also restrict some endogenous microbe growth too much such as NHglj1 under alkaline conditions, but the reasons and mechanisms are not clear.

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