The Study on Stress Resistance of Psammophyte *Agriophyllum squarrosum* Endophytic Diazotroph

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**Abstract.** In order to ascertain the growth of *Granulicatella elegans* GX(separated from lower root of *Agriophyllum squarrosum*), *Staphylococcus lentus* GZ(separated from middle root of *Agriophyllum squarrosum*) and *Sphingomonas paucimobilis* JZ(separated from middle stem of *Agriophyllum squarrosum* ) for *Agriophyllum squarrosum* (L.) endophytic diazotroph under different stress conditions, the test was conducted to explore effects of simulating desert salt, alkaline and high temperature on the colony diameter. The results show that ① NaCl tolerance range of *G. elegans* GX, *S. lentus* GZ and *S. paucimobilis* JZ is in 0.1 ~3%; GX strain can all survive in 5% NaCl, ② *G. elegans* GX, *S. lentus* GZ and *S. paucimobilis* JZ strains can grow well in pH 8～10, ③ *G. elegans* GX, *S. lentus* GZ and *S. paucimobilis* JZ have the better high temperature tolerance, and grow well in 28°C～38°C, GX strain can even survive in 42°C.

**Introduction**

Endophytic diazotroph can use excess energy produced by host plants to fix nitrogen in the process of plant tissue conduction and colonization, which is accepted macro regulation through plant control system[1,2]. Endophytic diazotroph embody higher nitrogen fixation efficiency, moreover, it can promote host growth by enhancing resistance of plants[3], especially it has a huge potential in solving plant nitrogen source in harsh desert environment [4], and enhancing the environment adaptability [5, 6]. The symbiotic relationship between endophytic diazotroph and plants has become one of hot topics in the field of Botany, Microbiology and Ecology [7,8]. Environmental conditions often influence colonization of endophytic diazotroph and fully promoting survive ability [9,10,11], so it is the key to effectively colonize in host body and establish combined relationship in harsh environment [12].The research on the symbiotic nitrogen fixation mechanism of plants and endophytic diazotroph has important theoretical and practical significance for understanding the plant adaptation and microorganisms in harsh environment [13,14].

*Agriophyllum squarrosum* is a cold resistance, drought resistance psammophyte, which has drought tolerance characteristic, saline-alkaline tolerance, sand burial, resistance to wind erosion so on[15], moreover, it is good shrub to be used as vegetation restoration and reconstruction, and is the highest nitrogen accumulation psammophyte. Existing studies have indicated that psammophyte may meet need by bacteria-plant symbiotic nitrogen[16].

At present, study on *Agriophyllum squarrosum* mainly involves in the vegetation succession, environmental adaptation and shifting sand fixation[17,18,19]. The study on endophytic diazotroph colonization on host plant and colonization increasing host plant resistance has frequently been reported, but less researches on endophytic diazotroph adaptability to stress have been reported. In this test, *Agriophyllum squarrosum* endophytic diazotroph is selected as test material to explore its...
saline-alkali tolerance and high-temperature resistance, which is to probe symbiotic relationship between endophytic diazotroph and host plant to provide evidences and references for biological nitrogen fixation under desert environment.

Materials and methodology

The tested nitrogen fixation strains are Granulicatella elegans GX (separated from lower root of *Agriophyllum squarrosum*), Staphylococcus lentus GZ (separated from middle root of *Agriophyllum squarrosum*) and Sphingomonas paucimobilis JZ (separated from middle stem of *Agriophyllum squarrosum*). These strains were classified and identified by Key Laboratory for Grassland Ecosystem of Ministry of Education and provided by Institute of Soil and Environment Bioremediation in Karst Habitats, Guizhou Normal College.

Primary situation of endophytic diazotroph separation

The study area is located in Xi Sha Wo (N38 degree E103 degrees 25 ') area, which belongs to the southeast margin of the BadanJilin Desert, north of 16 km of Minqin County, Gansu Province, flowing sand dune areas deep into 2 km desert, nutrient is poor. Extreme maximum temperature is 39.4 °C over the years, extreme low temperature is -28.8 °C, the average annual rainfall is 113.8 mm for many years, the average annual evaporation is 2604.3 mm, the days of annual average 8 degree gale is more than 37.7 d, the days of sand dust storm is 26.8 d, due to human over exploitation, underground water level has dropped below 20 m, desert vegetation degradation, degradation is serious, the ecological environment is very fragile [20].

The Configuration of Medium

Winogradsky’s modified nitrogen free medium [21], sucrose 10 g, malic acid 5 g, K$_2$HPO$_4$·H$_2$O 0.2 g, KH$_2$PO$_4$·H$_2$O 0.4 g, NaCl 0.1 g, FeCl$_3$ 0.01 g, Na$_2$MoO$_4$·0.002 g, MgSO$_4$·7H$_2$O 0.2 g (to be sterilized separately), CaCl$_2$·H$_2$O 0.02 g (to be sterilized separately), NaOH is used to adjust pH to 7.0±0.2, 1.5 % (mass to volume ratio) agar is added into the solid medium.

Salt, Alkali and High-temperature Stress Treatment

Winogradsky’s modified nitrogen free medium as basic medium was adjusted pH to 7.0±0.2, 1.5 % (mass to volume ratio) agar was added into the medium, the medium was sterilized with wet heat 26 min at 121°C, cooled at 80°C and added NaCl to adjust pH to detect strain resistance:

a. salt resistance medium: NaCl was added into basic medium to adjust salt concentration to 0.1%, 1%, 2%, 3%, 4% and 5% six stress gradients.

b. different pH environment free nitrogen solid plate: HCl and NaOH were used to adjust basic medium pH to 7, 8, 9, 10, 11 and 12 six gradients.

c. strains high temperature resistance test: the basic medium was separately loaded into erlenmeyer flasks, adjusting incubator temperature to 28 °C, 30 °C, 34 °C, 38 °C, 42 °C, 45 °C six gradients.

1.5% agar (mass volume ratio) was added into above medium, moist heat sterilization 26 min at 121°C, Agar plate was made in 9 cm culture dish after sterilization, Single colony growth of strains under different stress environment.

After the medium cooling and solidification, GX、GZ、JZ strain isolated from the lower end of root tissue was separately streaked in all treatment plates, 4 replications per strain. All salt resistance plates and alkaline resistance plates were placed into incubator to culture without light at 28°C, high temperature stress treatment plates were placed into multiple incubator, the treatment conditions was achieved by adjusting the temperature of the incubator.

After inoculation with 7d, single colony diameter for three strains under all treatments was measured by vernier caliper, 4 replications per treatment.

Data Procession and Analysis

The data was processed by EXCEL 2007 software and analyzed by SPSS 16.0 statistical software, LSD method, $P<0.05$. 
Results

The effects of salt stress on colony diameter of *agriophyllum squarrosum* endophytic diazotroph

Fig. 1 showed that different concentration NaCl has different effects on growth of three endophytic diazotroph, colony diameter for GX, GZ, and JZ decreased gradually with increase of NaCl concentration, moreover, GX, GZ, and JZ can grow at 0.1~5% NaCl,

![Fig. 1 The effects of salt stress on colony diameter of G. elegans GX, S. lentus GZ and S. paucimobilis JZ](image)

The colony continuing increase indicate that *agriophyllum squarrosum* endophytic diazotroph has certain tolerance to salt stress, this may be *agriophyllum squarrosum* endophytic diazotroph long-term adaptation to osmotic pressure, high concentration body fluid to form salt resistance. 1% or more NaCl has a significant inhibitory effect on single colony growth of three strains, colony diameter of three strains was significant small than that of control ($P<0.05$), and when the salt stress concentration gradually increased to 2%, colony diameter of JZ was slightly larger than the salt stress treatments of 1%, colony diameter of GX and GZ endophytic diazotroph were continuously decreased. The colony growth of JZ was severely inhibited, when the salt concentration increased to 3%, the colony growth was significantly less than that of 2% NaCl treatment, the similar trends was reflected in the salt concentration >4% for GX and GZ endophytic diazotroph.

On the other hand, all the strains were able to form colonies and continue to increase under 5% NaCl stress, this results indicated that different parts of *agriophyllum squarrosum* endophytic diazotrophs has good salt resistance. This may be that strain in order to adapt to high concentration fluid to form the resistance ability in the long term, but the specific adaptation mechanism has not yet been found in the molecular level of evidence, principles have yet to be further discussed.

The effects of alkali stress on colony diameter of *agriophyllum squarrosum* endophytic diazotroph

Fig. 2 showed that GX, GZ, and JZ can grow in pH 7.0~10.0, colony diameter of GX, GZ, and JZ decrease gradually with increase of alkali stress, colony growth was the most rapid in pH 7, the growth of the strain was significantly inhibited when the pH increased to 8, the colony diameter was lower than that of pH 7 ($P<0.05$), but the colony diameter of GX,GZ, and JZ strains increased significantly in pH 9, this results suggested that *agriophyllum squarrosum* endophytic diazotrophs was more sensitive to pH 8, pH 8 may also be used as a signal of stress regulation, which prompt the cell to adjust its metabolic process to make it more suitable for alkaline environment, for psammophyte *agriophyllum squarrosum*, light water deficit could induce the body fluid pH increased.
In the desert environment, the natural precipitation is concentrated, and the light water stress will cause the plant to reduce the intensity of metabolism to look forward to the arrival of another precipitation, nitrogen fixing bacteria in the body can also decrease the metabolic intensity while plants reduces the metabolic intensity. And pH further increased to 9, it may mean that water stress will gradually deepen, this usually indicates that the drought is coming, plants will face the gradual increase of sustained water stress, it means that *agriophyllum squarrosum* plant need to form grain as soon as possible to complete its life history. At this time, plant seed form, nitrogen demand increased, endophytic diazotrophs adapted to the desert environment with host plant, improved the metabolism activity of the cell to provide more nitrogen to the plant. When the pH increased, the metabolism and proliferation of the cell became slow, but the pH was 12, endophytic diazotrophs could still survive and reproduce.

![Fig. 2](image_url) The effects of alkali stress on colony diameter of *G. elegans GX, S. lentus GZ and S. paucimobilis JZ*

These results showed that *agriophyllum squarrosum* endophytic diazotrophs have better tolerance for alkaline, but it was pointed out that the proliferation and metabolic activity of the cell was also influenced and regulated by the environment pH, its specific mechanism was not clear, yet to be further studied.

The effects of high temperature on colony diameter of *agriophyllum squarrosum* endophytic diazotroph

Fig. 3 showed that the strains of GX, GZ, and JZ could grow at 28°C~38°C, all strains grew fastest in the 28 °C, with the increase of temperature, the colony diameter decreased continuously, these results showed that *agriophyllum squarrosum* endophytic diazotroph had wide range adaptation to temperature, at the same time, it was found that the growth rate of colonies for GX, GZ and JZ was the fastest in the non nitrogen solid culture medium, the reproduction rate of three strains of endophytic diazotroph was also close to that of the temperature. At 30 °C, the plant's cell proliferation was inhibited. But the three representative strains were able to withstand the 38°C, this was the common high temperature stress in summer and autumn day sand surface of desert areas for *agriophyllum squarrosum*. Under this condition, the diameter of single colony could still increase, but the colony diameter was significantly less than that of at 28 °C ($P<0.05$). It was noteworthy that GX could withstand 42°C, this may be related to living area survival of plant in the long-term, root system of *agriophyllum squarrosum* distribute in superficial dune slope, the root system was slender, and the distal end was distributed in the lower part of the moving dune. Compared with stem and root system,
the lower end of the root was more concentrated in the lower part of the sand dune with better water condition. Also in the daytime sand surface high temperature, the water condition in the lower part of the root system was better, and the heat resistant ability of the cell in root tissue was stronger, above results indicated that *agriophyllum squarrosum* endophytic diazotroph generally had good tolerance to high temperature, and its resistance was closely related to the location of the plant body and the microstructure of the plant.

![Image](image.png)

**Fig. 3** The effects of high temperature on colony diameter of G. elegans GX, S. lentus GZ and S. paucimobilis JZ

**Discussion**

In terms of salt stress, there were some differences in the salt tolerance between endophytic diazotroph of the stem and root, the endophytic diazotroph of the stem was better in salt stress tolerance of 2%, above this concentration, the colony growth was severely inhibited, and the endophytic diazotroph of root were more sensitive to the low salt stress of 1%, however, with the increase of salt stress concentration, the strain growth inhabitation trend became slow, this phenomenon may be related to the survival strategy of the host in the desert, that was *Agriophyllum squarrosum* usually adopt drought avoidance strategy, the moisture in the soil of the root was sufficient, plant growth was the most vigorous, nitrogen requirement was also the highest. At this time, the intracellular fluid concentration was relatively low, roots as the source of water absorption, the osmotic stress of nitrogen fixing microorganism in the root was lighter than that of in the stem. Nitrogen fixation effect of nitrogen fixing bacteria was obvious. Compared with root system, plant stems and leaves have larger evaporation and higher cytosol concentration, the nitrogen fixing bacteria in the stem need to elaborate the strong nitrogen fixation to maintain higher resistance penetration ability. When the water content in the root soil decreased significantly, the desert was in drought period, *Agriophyllum squarrosum* plants quickly enter reproductive growth to complete their life cycle. Leaves first dehydrate and dry, the stem lost water and dry from outside to inside while the stem continues to transport nutrient to grain, during this process, azotobacter could still play the role of nitrogen providing at first, however, the effect of nitrogen fixation still consume photosynthetic products of plant accumulation, with the continuous increase of cytosolic concentration of stem tissue, the reproduction and metabolism of azotobacter were severely inhibited, nitrogen fixation decreased, the economical photosynthate in the plant was transported to grain until stalk dehydrated and lost transportation function, at this time, the nutrient transport channel was closed due to loss of water,
sugars in *Agriophyllum squarrosum*’s roots were difficult to transport to the grain of the ground, *Agriophyllum squarrosum* did not germinate and breed again through the root, there was no need to store nutrients waiting for germination again, at this time, the water condition of the root system was also slightly better than the above ground part, so the remaining nutrients could be used by azotobacter, on the one hand, the azotobacter proliferate cells in order to maintain the existence of strains, On the other hand, the azotobacter could also store nitrogen to be utilized by the next generation *Agriophyllum squarrosum* plant.

The above corollary could be proved from the changing trend of soluble sugar content in *Agriophyllum squarrosum* plant, from vegetative growth stage, reproductive growth preliminary stage, reproductive growth final stage to age of the dead body, the body fluid concentration of *Agriophyllum squarrosum* stalk continued to increase, and the soluble sugar content decreased sharply, after the reproductive growth final stage, the soluble carbohydrate increased in roots and stalks of other non desert or perennial plants, but there was not much soluble carbohydrate left in stem tissues of *Agriophyllum squarrosum* plants, the soluble carbohydrate in the root system was also far below the reproductive growth preliminary stage (data published in another article).

It was thus clear that the difference between salt tolerance of different parts of *Agriophyllum squarrosum* and salt concentration change probably meant endophytic diazotroph reproduction, metabolizing, nitrogen fixing in *Agriophyllum squarrosum* existing to osmotic adjustment as the basis of the efficient regulation mechanism, when the water and light were enough, metabolism of azotobacter increased under low body fluid concentration, the amount of nitrogen fixation increased, it was beneficial to rapidly accumulate photosynthate, protein and other nitrogenous products. When the water was short, plants need as much as possible to transfer enough plant nutrients to grain, rapid dehydration caused the stem, leaves, and all unnecessary tissue to dry and die in order to reduce energy consumption of metabolism, the stem internal organization with only retaining transport function reduced metabolism and nitrogen fixation ability because body fluid concentration increased, reduced or closed the cell nitrogen fixation process, the ability of azotobacter resistant to high concentrations osmosis press in the roots was stronger than that of in the stems, the nutrients which were not be transported to the grain in root tissue could be better used to maintain the survival of the cell.

In this test, GX could even withstand 42°C high temperature, on one hand this result indicated that *Agriophyllum squarrosum* plant could adapt to desert environment, its endophytic diazotroph also formed resistance for salt, alkali and high temperature; this might be endophytic diazotroph and its host plant could gradually form a synchronization correspond mechanism under harsh environment; on the other hand, the emergence of high resistant strains of endophytic diazotroph could provide the strain material for the special microbial fertilizer in the desert area, moreover in harsh environment, adaptable plants as host plant was microorganism realizing mutually beneficial relations between with plant through its function, it was one of the strategies to sustain life.

**Conclusions**

In this research, three tested strain *Agriophyllum squarrosum* endophytic diazotroph-G. elegans GX, S. lentus GZ and S. paucimobilis JZ could separately grow in 5% NaCl, in pH 12, in 38 °C high temperature,even GX can even withstand 42°C high temperature, these results suggested that *Agriophyllum squarrosum* endophytic diazotroph had higher salt, alkali and high temperature resistance, meanwhile, *Agriophyllum squarrosum* endophytic diazotroph and its host plant could form a synchronization correspond mechanism to resist stress under harsh environment. The application of *Agriophyllum squarrosum* endophytic diazotroph in other desert plants is worthy of further study, which can not only provide natural nitrogen fertilizer, but also enhance the tolerance of host plants to stress.
Conflict of interest
The authors declared that they have no conflicts of interest to this work.

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