A Study on *Kalanchoë daigremontiana's* Viviparity under Drought Stress

- A Potential Anti-desertification Plant

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Content

1 Abstract

Accounting for a vital part of ecological environment, drought can lead to significant consequences, such as dust storms. *Kalanchoe daigremontiana*, also called 'Mother of Thousands' or 'Mexican Hat Plant', is a succulent plant native to Madagascar, which can endure severe drought. This research focused on *Kalanchoe daigremontiana*'s responses to drought stress and the mechanism of its response, especially paying attention to its unique vegetative reproduction, "viviparity".

Plants were given drought stresses of different levels. Besides observational experiments, the research also covered relative water content, protein concentration, Catalase and Peroxidase activities, hormone level measured, as well as RNA level of certain related gene. Methods used include spectrophotometer, chromatography, RNA extraction, reverse transcription and Real-time PCR.

Among all reactions observed, it is most impressive that drought can promote *K.daigremontiana*'s reproduction. With various experiments set for studying the mechanism of this promotion, it is concluded that the environmental stress exerted on *K.daigremontiana* causes the plant to release abscisic acid, which then through a bZIP-and-ABRE-related-pathway regulates *kd*LEC1, a gene related with *K.daigremontiana's* viviparity.

Kalanchoe daigremontiana itself may improve soil condition conveniently, quickly, aesthetically, and economically. Furthermore, if studies on other viviparous species have similar results, non-viviparous species may be genetically modified to be viviparous by genetic engineering, with enhanced drought endurance and increased reproduction speed under drought.

Key Words: Kalanchoë daigremontiana, environmental stress, drought stress, viviparity, abscisic acid, catalase, peroxidase, relative water content, protein content, High Performance Liquid Chromatography, RNA extraction, reverse transcription, Real-time PCR, Abscisic acid responsive element

2 Introduction

Soil condition accounts for a vital part of ecological environment. Soil under drought not only cannot sustain the soil structure but also may result in sand storm and other air pollution problem. It can be pointed out that there exist such pollution problems in soil quality, which should grip our alert and attention.

Under different soil conditions, plants' growth and reproduction conditions vary. Sometimes, soil with certain pollution will form a kind of environmental stress on the plants, laying stress on the plants' living situation and causing them to change.

It has been reported that plants in the Crassulaceae family have the characteristic of heavy metal accumulation and drought endurance, which can provide possible usage in improving soil quality. *Kalanchoë daigremontiana* is one of the plants with such characteristics, catching our attention with another unique feature: a special reproduction procedure, viviparity.

K.daigremontiana, originated from Madagascar, is commonly known as the "mother of thousands" or "Mexican Hat Plant", reproducing by viviparity, which means it forms somatic embryos outside a seed environment. Plantlets develop along leaf margins, and then fall to the groud to grow into new plants. Plant extracts from some *Kalanchoe* species were shown to have anti-tumor, anti-inflammatory, and insecticidal properties.

If a small number of *Kalanchoë daigremontiana* are put in a polluted area, soon it will reproduce huge numbers of new plants with leaf-plantlets. Such a rapid reproduction may help improve the soil quality both quickly and conveniently with low cost. Such unique quality rouses our interest. Though morphological and genetic aspects of its viviparity have been carefully studied, no previous research referred to its responses and potential uses under drought. Thus, we chose it as our experimental material.

Our research focused on how and why the reproduction of this plant is influenced by different soil conditions, and discussed the possibility to take advantage of it so as to improve soil quality. Observational experiments were conducted, inner measurements of the plants tested. It was then proved that drought condition may in fact promote the reproduction procedure of *K.daigremontiana*, and the mechanism was later studied.

From our results, *K.daigremontiana* reproduces even better under drought or heavy metal conditions. This means, in order to use *K.daigremontiana* for improving soil quality, only a small number of plants are required, because by reproducing themselves quickly under such conditions through viviparity, they can soon cover a large area. Thus, growing *K.daigremontiana* to improve soil quality would not only be an effective method, but also an economic one.

3 Materials and Methods

3.1 Treatments

Plants were kept in a growth-cabinet. Temperature was controlled as 25C during the day and 19C at night. Day length was controlled as 12h/day, and relative humidity was 60%.

All plants were divided into three groups; each of them was given different treatment separately.

Drought: Plants were watered at fixed time during the day, once every three days. Each time, the amount of water supplied for corresponding groups were accordingly 0ml, 5ml, and 10ml. These were accordingly the heavy drought group, moderate metal group and the control group.

Heavy Metal (Cu^{2+}): Plants were grown in nutrient solutions with different amount of $CuSO_4$. The concentration of Cu^{2+} in control groups were accordingly 0mg/L(control group), 5mg/L, 10mg/L, and 25mg/L.

Extraneous Abscisic Acid: Plants were grown in nutrient solutions with and without 0.3mmol/L ABA.

Determine leaf relative water content to verify the drought stress previously given to the plants.

3.2 Measurements

Measurement of Leaf Relative Water Content:

A sample of leaf is taken, and the fresh weight is determined. Then the turgid weight is then recorded by soaking the sample in water until a constant weight. The leaf tissue is subsequently over-dried to a constant weight at about 85C.

Relative Water Content is calculated by

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}}$$

Measurement of Protein Concentrations:

100mg Coomassie brilliant blue G-250 was dissolved in 50ml 95% ethanol. Then, 100ml 85% phosphoric acid was added. The mixture was diluted with H2O to 1000ml.

BSA (Bovine Serum Albumin) was used as calibrator. BSA protein assay solution of the following concentrations was made: 0mg/ml, 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, 1mg/ml. 0.1ml of the solution was mixed with Coomassie brilliant blue mentioned above. After 5min ~ 1h, the absorbance of the assay mixture was measured by a spectrophotometer. A calibration curve was calculated.

Leaf tissue (0.2g) was crushed in an iced mortar and pestle with 5ml of pH 7.0 phosphate buffer. The homogenate was centrifuged at 4000r/min for 10 min. The supernatant was transferred to a 10ml volumetric flask. 2ml phosphate buffer was added to the residual, and centrifuged again at 4000r/min for 10min. The supernatant was combined to 10ml.

The following procedure of measuring was the same as that of BSA protein assay solution.

Protein concentrations were derived by extrapolation of the absorbance values from the calibration curve.

Protein concentration (μg/mL)	0	200	400	600	800	1000
Absorbance	43.8%	35.4%	29.1%	21.6%	15.6%	9.7%

Table 1. The calibration curve

Measurement of Catalase and Peroxidase Activities:

Leaf tissue (0.25g) was crushed in an iced mortar and pestle with 5ml of pH 7.0 phosphate buffer. The homogenate was centrifuged at 15000r/min for 15 min. The supernatant was used for assays of peroxidase and catalase activities.

For peroxidase, 3ml of assay mixture contained 0.3% H_2O_2 1ml, pH 7.0 phosphate buffer 1ml, 0.2% guaiacol 0.95ml, and 0.05 ml of enzyme. The rate change of optical density at 470nm was measured using a spectrophotometer.

For catalase, 3ml of assay mixture contained 0.3% H_2O_2 1ml, H_2O 1.95ml, enzyme 0.05ml. The rate change of optical density at 240nm was measured using a spectrophotometer.

Extraction and Measurement of Endogenous Abscisic Acid:

Endogenous level of ABA in leaves was measured by high-performance liquid chromatography. Calibration curve was calculated with (±)-Abscisic Acid.

Chromatographic column: Agilent Eclipse Plus C18

Mobile phase: A 10% acetic acid, B methanol, (A/B = 55/45)

Flow rate: 1.5ml/min

Wave length: 254nm

Leaf tissue (10g) was crushed and mixed with 100% methanol. After 24h, the mixture was filtered with suction filtration. Then, the solution was evaporated with rotary vacuum evaporator at 40C, 70r/min. The concentrated liquor was extracted with ethyl acetate for 3 times, at pH2.0. After decoloring with silica gel, the solution was eluted with chloroform: ethyl acetate: acetic acid = 5: 4: 1.

The solution was measured similarly with the calibrator.

Extraction of Total RNA

Two methods of extractions were tried. Extraction with a method of precipitation (Garces et al. 2009) did not work well, with a total RNA yield of only 0.032 ug/uL. Extraction with Qiagen RNeasy Plant Mini Kit can achieve a satisfactory total RNA yield of 0.432 ug/uL. The extraction followed the kit's instruction except that 0.1M PEG-20000 was added to the RLC buffer. (Gehrig HH et al. 2000)

RNA concentration and purity were determined by measuring the ratio of UV absorbance at 260nm and 280nm.

Reverse Transcription

RNA was reverse transcribed into cDNA, and the cDNA was then used as the template for Real-time PCR amplification. With TaKaRa PrimeScript RT reagent kit (TaKaRa code DRR047A), gDNA was first erased before reverse transcription. The kit's instructions were carefully followed. The reverse transcription was done in a bio-rad Thermal Cycler C1000.

Real-time PCR

Real-time detection of PCR products is enabled by SYBR Green I. The amplification plot shows two phases, an exponential phase followed by a non-exponential plateau phase. During the exponential phase, when enough amplified product accumulates to yield a detectable fluorescence signal, the cycle number is called the C_T value. The $2^{-\Delta\Delta C_T}$ (Livak) Method was adopted for data analysis.

The target gene was LEC1 with primer：5′-GCCAGCATGAGCAGAGGAAGACC-3′，5′-GCCCACAGCCATTCCTATTCCCATTC-3′. The reference gene was GAPDH with primer: 5′-GG′, 5′-TCCATTCATCAACACAGACTAC-3′.

Real-time PCR was performed in ABI PRISM 7500 Fast Real-Time with TaKaRa SYBR Premix Ex Taq Kit (TaKaRa code DRR420A).

The cycling program used is shown below:

Cycle 1	1 repeat	95 **°C** for 30 sec
Cycle 2	40 repeats	95 **°C** for 5 sec, 55 **°C** for 30 sec, 72 **°C** for 60 sec

Besides the amplification curve, a melt curve is also created to see whether the product is the expected one. The cycling program for melt curve:

Cycle 1	1 repeat	95 **°C** for 30 sec
Cycle 2	40 repeats	95 **°C** for 3 sec, 60 **°C** for 30 sec

Hypothesis Testing

All data have been tested for its significance in statistics. Hypothesis testing is used. P-value testing is been chosen. P-value represents the probability that such extreme case as the observed data will occur, given that the null hypothesis is true. If $p < 0.05$, it means the data show significance in statistics; if $p < 0.01$, it means the data show great significance in statistics.

4 Results

4.1 *Kalanchoe daigremontiana*'s Responses to Drought Stress

4.1.1 *K.daigremontiana*'s Viviparity

K.daigremontiana showed certain tolerance towards drought stress. Its reproduction process, viviparity, was better under drought compared with that of the control group, attaining a very significant degree ($p<0.01$). Furthermore, plants treated with heavy drought sprouted leaf-plantlets even more.

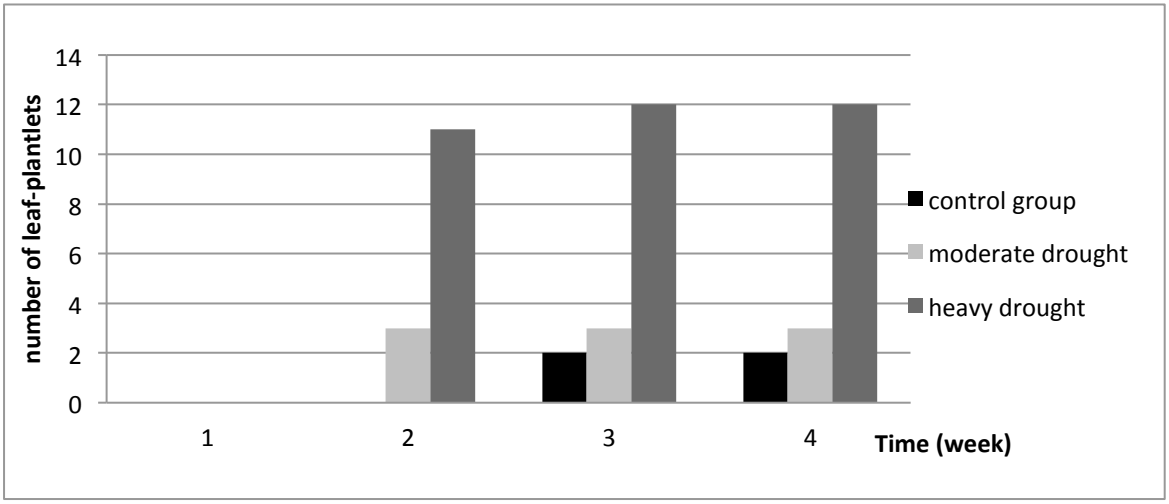

Fig. 1 Average number of leaf-plantlets under drought condition

4.1.2 Stems and Leaves

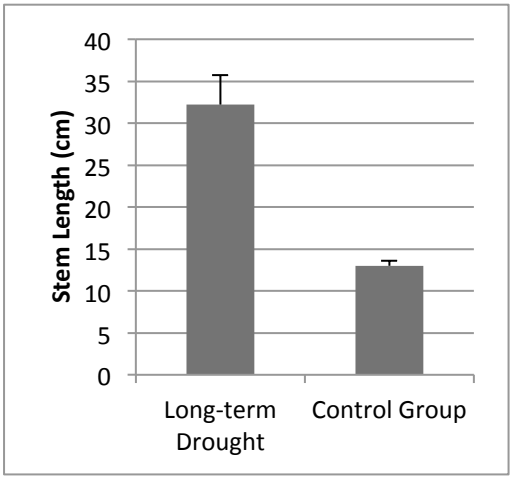

Fig. 2 Average stem length

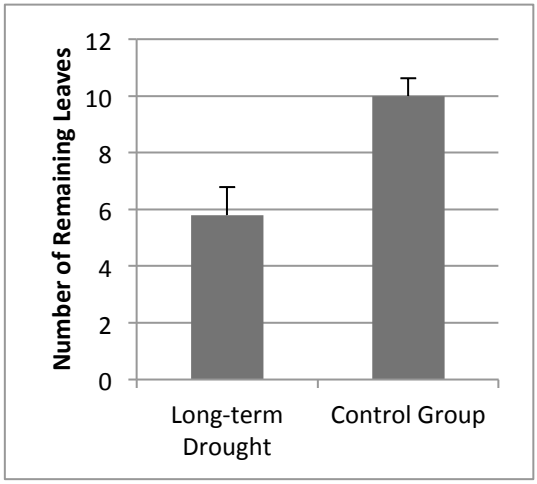

Fig.3 Average number of remaining leaves (per plant)

After being in a drought condition for two months, plants grew taller significantly, with their stems becoming thinner. Aerial roots were also observed on the stems after drought treatment. It is possible that *K.daigremontiana* grow longer stems in order to reach a piece of ground with more water resources and then take root with the aerial roots.

It was also observed that leaves withered and fell under drought. Only approximately half of the leaves remained. Plus, remaining leaves were smaller and lighter.

4.1.3 Protein Concentrations

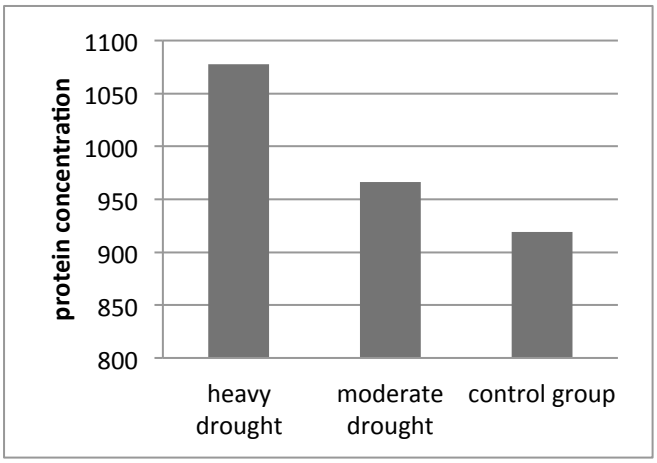

Fig.4 Protein concentrations under drought condition

The result showed that protein concentrations of drought groups were higher than that of the control group, and protein concentration increased with the level of drought.

4.1.4 Catalase and Peroxidase Activities

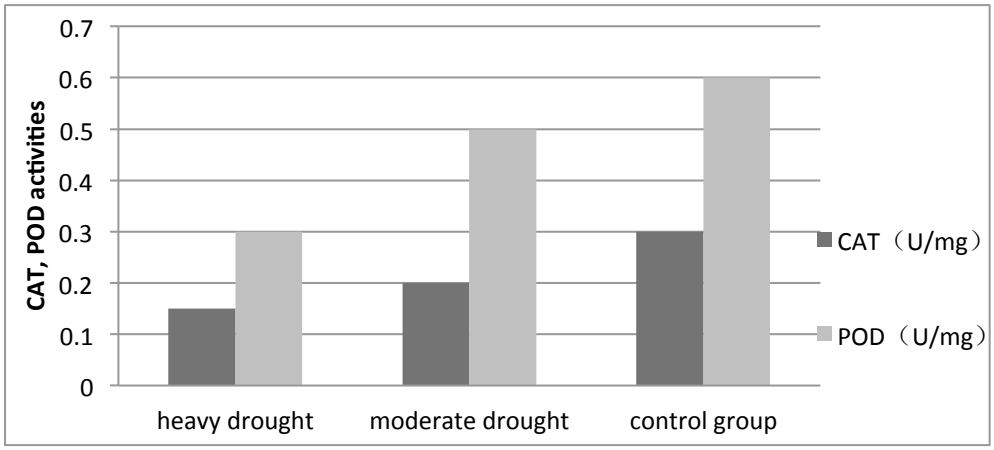

Fig.5 CAT, POD activities under drought condition

The result showed that Catalase and Peroxidase activities in the drought groups decreased with increasing level of drought stress. If using protein content as unit, such trend is magnified and becomes even more significant.

4.1.5 Leaf relative water content

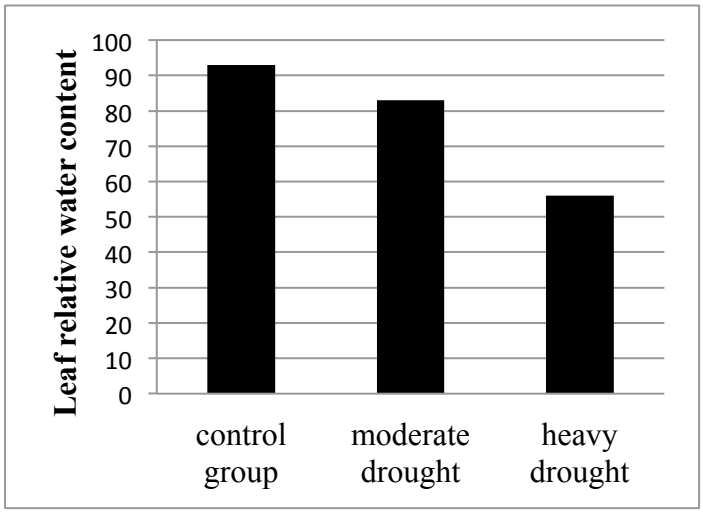

Fig.6 Leaf relative water content

In figure 6, it can be concluded that in the drought stress group, with the increase of the watering quantity every two days, the relative water content increases and showed a significant degree ($p<0.05$). It accords with the initial experimental setting.

4.2 Abscisic Acid (ABA)

Among all responses, it is especially interesting and exciting to see *K.daigremontiana*'s increased viviparity under drought stress, due to its important meaning in improving soil quality in dry areas. The rest part of this research focused on studying the mechanism of such promotion. As ABA is widely acknowledged as related with drought stress, experiments were designed to investigate the role of ABA.

4.2.1 Endogenous ABA Level

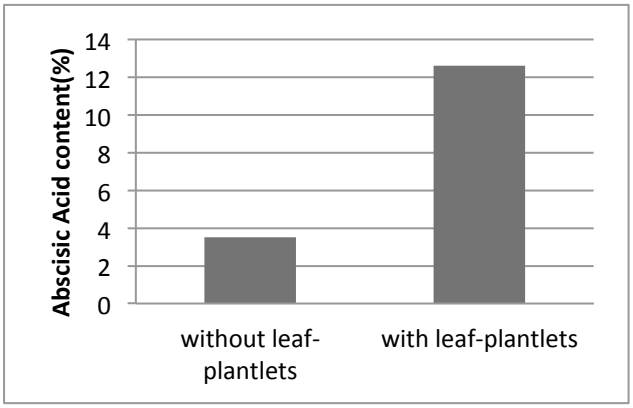

Fig.7 Relative Abscisic Acid content under drought, using the area normalization method to directly reflect the percentage of content (take total area as 1)

The abscisic acid content of those with leaf-plantlets is more than that of those without leaf plant-lets, indicating that ABA might be related with the promotion of *K.daigremontiana*'s reproduction under drought condition.

4.2.2 *K.daigremontiana*'s Viviparity with Extraneous ABA Treatment

It can be shown in Fig.7 that those plants with certain amount extraneous abscisic acid treated sprouted more leaf plant-lets, attaining a very significant degree ($p<0.01$). That is to say, ABA can induce *K.daigremontiana*'s viviparity.

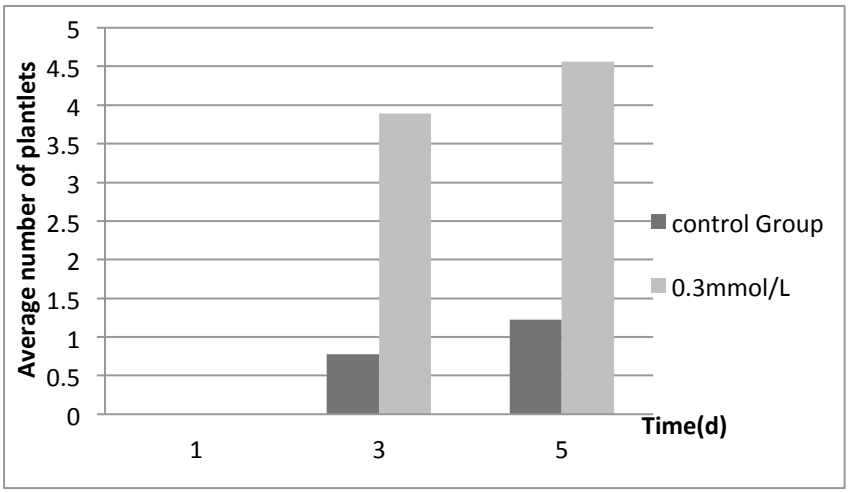

Fig. 8 Average number of plantlets under 0.3mmol/L ABA treatment and control group

4.3 Further Discussion in Mechanism

4.3.1 *kdLEC1* (Leafy Cotyledon-like 1 gene) RNA Levels

With methods described before, average experimental results were as following.

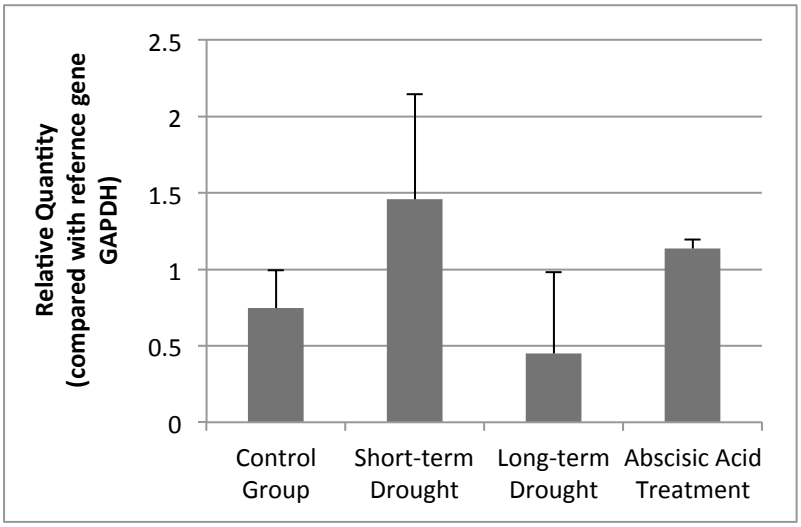

Fig. 9 Real-time PCR result of *kdLEC1* RNA relative quantity under different treatments

K.daigremontiana's *kdLEC1* is related with its viviparity (Garces, 2007). As shown in Fig.8, 2-week drought treatment or 2-week ABA treatment can increase the quantity of *kdLEC1*, while a 2-month drought treatment lowered *kdLEC1* quantity.

4.3.2 ABREs in *LEC1* sequence

An ABRE (abscisic acid responsive element) functions as a *cis*-acting element involved in ABA-regulated gene expression. ABA activates bZIP proteins to bind to ABRE and initiate transcription of ABA-inducible genes. Different ABREs have been identified, such as ACGTGGC. There are *LEC1* sequences of 12 *Kalanchoe* species available, in which 8 ABREs are searched for throught BLAST.

Table 2. ABREs found in LEC1 sequence. 3 out of 7 ABREs were found.

Element	
ACGTGTC	*k.daigremontiana LEC1* (180~186)
ACGTTTC	*k.daigremontiana LEC1* (1388~1394) *k.pinnata LEC1* (355~361) *k.beauverdii LEC1* (282~288)
ACGTGGC	*k.daigremontiana LEC1* (183~177)

4.4 *K.daigremontiana*'s Viviparity under Other Stress

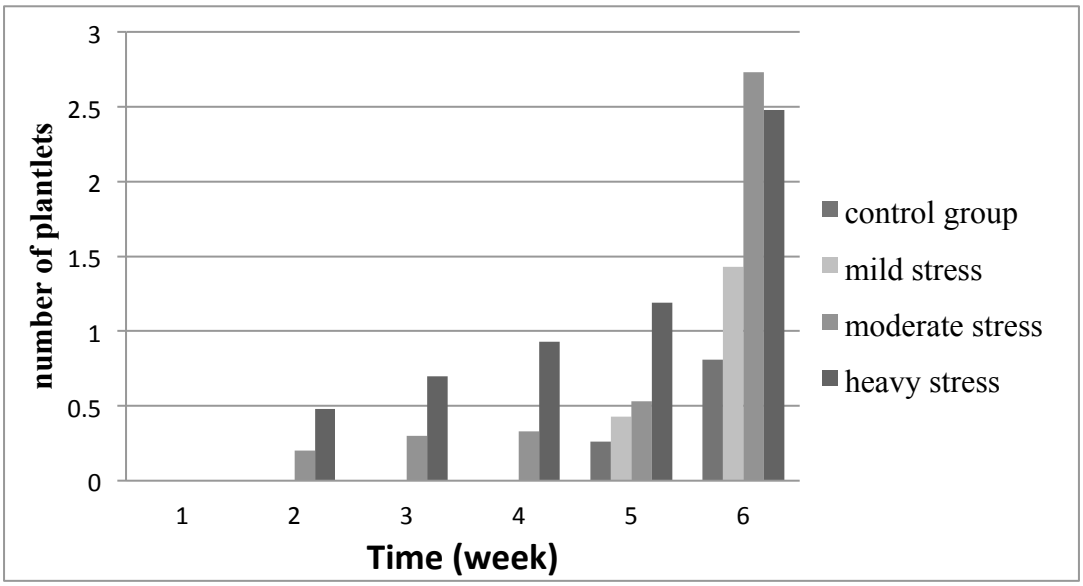

Fig. 10 Average number of leaf-plantlets under Cu^{2+}condition

As to see whether other environmental stresses may have similar effect *on Kalanchoë daigremontiana,* we chose heavy metal stress. If Cu^{2+} concentration did not exceed 25mg/L (Experiments and documents show that such concentration will not seriously affect the survival of the plants), the plant sprouted more leaf-plantlets. Its viviparity under soil with heavy metal was better than those from the control group, attaining a very significant degree ($p<0.01$). In addition, such promotion was more significant as Cu^{2+} concentration increased. It is possible that other stresses such as heat stress, salt stress or cold stress can also promote its viviparity.

5 Discussion

This research focused on *K.daigremontiana*'s responses to environment stress, especially paying attention to its viviparity, and discussed the mechanism of various responses, thus providing a method of bioremediation.

The major aspect studied was *K.daigremontiana*'s viviparity. Statistically analyzed, *K.daigremontiana*'s viviparity is significantly promoted under drought stress. This research is the first ever to discover such kind of promotion.

Then, the research focused on the mechanism of the promotion mentioned above. *K.daigremontiana*'s endogenous ABA level increased in leaves with leaf-plantlets on margins after enduring drought. It is widely acknowledged that ABA acts an important role in plant water stress responses. With experiment, we verified that ABA can induce the plant's viviparity, as it developed more plantlets as well under extraneous abscisic acid (ABA) treatment.

Further experiments were set to study even deeper mechanisms. Leafy cotyledon 1 (*kdLEC1*) is a gene reported to be strongly related with *K.daigremontiana*'s viviparity (Garces, 2007). It was also discovered in our research that *kdLEC1* increases in quantity under drought stress and extraneous ABA treatment, thus indicating that both drought and ABA treatment can induce kdLEC1, which then controls *K.daigremontiana*'s viviparity.

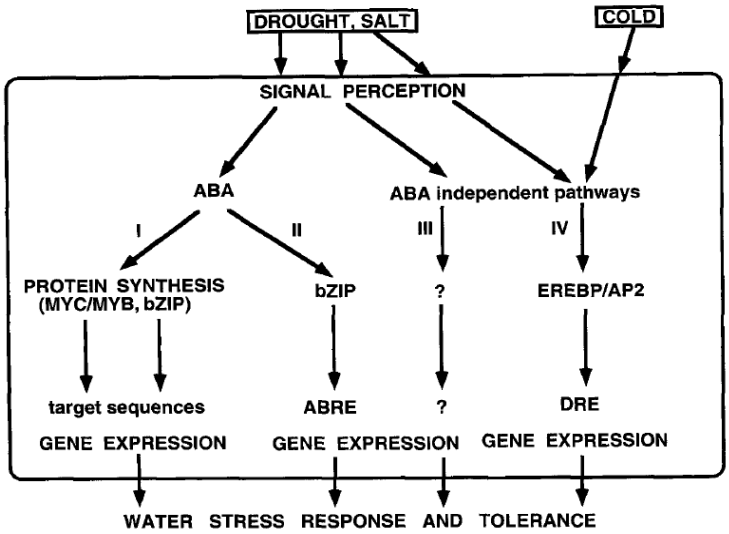

Fig. 11 Signal transduction pathways between perception of a water-stress signal and gene expression

(K.Shinozaki, 1997)

As shown in Fig. 11, at least four signal transduction pathways exist (I-IV): two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein biosynthesis is required in one of the ABA-dependent pathways (I). In another ABA-dependent pathway, ABRE does not require protein biosynthesis (II). In one of the ABA-independent pathways, DRE is involved in the regulation of genes not only by drought and salt hut also by cold stress (IV). Another ABA-independent pathway is controlled by drought and salt hut not by cold.

It can be easily noticed that one of the major differences between Pathway I and II is that target genes in pathway II include ABRE (ABA responsive element) in their promoter, while target genes in pathway I do not. For kdLEC1, two ABREs are found in its promoter sequence: ACGTGTC and ACGTGGC. Thus, it should be deduced that *K.daigremontiana*'s viviparity is influenced by drought through ABA-bZIP-ABRE-kdLEC1.

Besides viviparity, the influence of drought on other indicates and characteristics were also carefully studied.

First of all, relative water content (RWC) is chosen as parameters indicating plant water status. Water potential has gained wide acceptance as a fundamental measure of plant water status. Relative water content is another commonly used indicator of plant water status. (Hsiao, 1973) With the increase of drought stress, *K.daigremontiana*'s leaf water potential increases, and relative water content decreases, both verifying the drought stress.

Drought stress can cause *K.daigremontiana* grow longer stems. Aerial roots were also observed on the stems after drought treatment. It is possible that *K.daigremontiana* grow longer stems in order to reach a piece of ground with more water resources and then take root with the aerial roots. Growing aerial roots might also serve the purpose of absorbing water from air.

Leaves react to the environmental stress by withering and falling, along with decreased number of stomata. It is well documented that stomatal closure is the main cause for transpiration decline as water stress develops. (Hsiao, 1973)

Protein concentrations increased under drought. Protein in plants mostly is enzyme participating in metabolic, making the content of protein an important index to know the whole metabolic level of a plant. However, when specific enzyme activities were examined, the Catalase and Peroxidase activities decrease with the increase of drought level. It was concluded that severe stress generally lowers enzyme levels although often raises the levels of enzymes involved in hydrolysis and degradation. (Todd, 1972)

It is very interesting to see these changes in response to drought stress. It could be concluded that *Kalanchoe daigremontiana* grows and reproduces surprisingly well under drought conditions through various responses, making it a suitable candidate for bioremediation of dry soil and a potential anti-desertification plant.

6 Conclusion

Drought stress can promote the viviparity of *K.daigremontiana*, and promotes the its viviparity in the following way:

1. The soil condition causes environmental stress on the plant. 2. The plant releases ABA (abscisic acid). 3. ABA regulates *kd*LEC1 through a bZIP-and-ABRE-related pathway. 4. *kd*LEC1 controls the growth of leaf-plantlets.

Besides viviparity, under drought condition, *K.daigremontiana* develops longer stems and aerial roots. A large percentage of leaves fall, and stomatal closure is observed. Protein concentration increases, while Catalase and Peroxidase activities decrease.

K.daigremontiana can endure severe drought through various adaptations mentioned previously in this research. If planted in certain areas with drought, it may improve the soil quality through rapid reproduction. From our results, *K.daigremontiana* reproduces even better under drought or heavy metal conditions, which means, by planting a small number of them and letting them reproduce quickly through viviparity can a large area soon be covered. Thus, growing *K.daigremontiana* to improve soil quality would not only be an effective and aesthetic method, but also an economic and convenient potential anti-desertification method.

In the future, the research will possibly extend in the following aspects: to plant and experiment *K.daigremontiana* in fields, to study other environmental stresses and to study other viviparous species.

There are other viviparous species. If the soil conditions have a similar effect on viviparity in other plant species, those species may have various potential usages under drought. Furthermore, non-viviparous species may possibly be altered to have this amazing reproduction procedure by genetic engineering, with their reproduction speed under drought dramatically increased.

Acknowledgement

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