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BANANA (*MUSA*. SPP.) STRAIN 'HD-1' APPRAISAL

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Abstract

Being one of the important tropical and subtropical fruit trees, banana (*Musa.*spp.) belongs to the family *Musaceae* and the order Scitaminae with two genera, *Musa* and *Ensete*. In a field survey, research team has discovered a potential banana mutant strain 'HD-1' with a sound economic value. The results of the finding are as follows: based on Simmonds' classification, the pseudostem of banana strain 'HD-1' is relatively short and purplish red; its upright outward petiole groove has red edges and wraps its pseudostem loosely. Its ploidy is 3×, AAA type. Karyotype analysis shows that the number of chromosomes is 33, the karyotype formula is 2n=3x=33=2L + 3 M2 + 4 M1 + 2 S, 'HD-1' is classified as '1B' type. With the help of ISSR molecular markers, we find thatbanana 'HD-1' has the closest relationship with Pubei and Tianbao dwarf banana; the similarity coefficient is 0.81. In an artificial simulation tests of cold, drought and salt resistance environment changes of physiological and biochemical indexes indicate that 'HD-1' exhibits stronger defense capability than Brazil banana. By way of inoculation with injury of root dipping method, we respectively treat two kinds of banana seedlings inoculated Banana Fusarium wilt race 4 small species. The results show that their resistance evaluation scores are 3 and 4, disease levels are 'susceptible' and 'high sensitivity' respectively. We conclude that 'HD-1' has stronger resistance ability toFusarium wilt than Brazil banana.

Key words: Banana strain 'HD-1'; Genetic marker; Resistance; Stress.

Introduction

Banana (*Musaceae, Musa*) is one of the competitive products in China. Banana tastes sweet and is highly nutritious.It is rich in carbohydrates, dietary fiber, protein, fat and vitamins A, C and B_6. In fact, banana is one of the earliest crops cultivated in the history of human agriculture. The origin of this particular plant family stretches from India to Papua New Guinea including the Southeast Asian region (Padam *et al.*, 2014). India, China, Uganda, Ecuador, Philippines, and Nigeria are deemed as the world leading banana and plantain producers. Banana has grown in over 130 countries; mostly in the tropical and subtropical areas and has a centre of origin from South-East Asia. In economic terms, it is the fifth agricultural food crop in terms of world trade, after coffee, cereals, sugar and cacao; and is an important fruit crop apart from grapes, citrus fruits and apples (Aurore *et al*., 2009). Banana is a very popular fruit in the world market ranking next to rice, wheat and maize in terms of its importance as a food crop(Singh *et al*., 2016).The annual yield of banana is approximately 100 million tons worldwide, and more than 1.2 million tons in China alone. Guangxi is one of the largest banana-producing provinces in China with banana plantations located from the southeast to southwest regions (Lin *et al*., 2011). Banana industry plays an important role in tropical economy and rural social development. If commercial banana varieties degrade, we are unlikely to have an alternative variety to meet the diverse ecological conditions and to reserve different market needs. For example, in November 10, 2013, 'Swallow': a strong typhoon, brought devastating destructions to Hainan Province. Bananas in Sanya, Ledong and other regions were destroyed, causing severe economic loss（http://news.xinhuanet. com/politics/2013-11/12/c_125689419_6. htm). The main genomic groups are AA, AAA, AAB and ABB, although AB, AAAB, AABB and ABBB are also possible. Highly related clones or cultivars resulting from mutations in a single genotype are allocated to so-called subgroups, characterized by specific morphological and fruit quality attributes (Creste *et al*., 2003).

The banana industry worldwide has undergone several pathogens and pests outbreaks, and due to the lack of suitable resistant varieties, its chemical treatments have been widely adopted as the only control strategy (Creste *et al*., 2004). The genetic complexity in the genus *Musa* has been subject of study in many breeding programs. Parthenocarpy, female sterility, polyploidy in different cultivars and limited amount of genetic as well as genomic information makes the production of new banana cultivars difficult and time-consuming (Capdeville *et al*., 2009). Most banana cultivars reproduce asexually, and some varieties are believed to have been in cultivation for up to 8,000 years (Nair *et al*., 2005).

Countries concerned have been taking pains to collect, conserve, evaluate and utilize banana germplasm. In 1985, the International Network for the Improvement of Banana and Plantain (INIBAP) was established in the University of Leuven in Belgium under the help and supervision of the International Plant Genetic Resource Institute (IPGRI), whose purpose is to provide assistance for studying, evaluating, improving and promoting new varieties of the global banana germplasm resources; and to facilitate research on banana in different countries and regions. International *Musa* germplasm collections established to preserve native accessions from wild species and cultivated clones encourage international exchange of indexed germplasm that provides support to some recognized banana breeding programs (INIBAP 2006). In China research on *Musa* germplasm resource started early in 1950s, however the progress was slow till the twentieth century. After the 80's, protection and utilization of banana germplasm resource enjoyed a rapid development. In 2008, Tropical South Asia Tropical Tree Branch of China Horticultural Society set up a banana

Network. The network not only promotes protection and utilization of banana germplasm resources in China, but also reinforces communication and cooperation between workers in this country and their counterparts elsewhere in the world. As M. N. Normah *et al*. (2013) pointed out that the enetic diversity of tropical fruit trees is increasingly under threat; in the case of cultivated species by specialization of production systems in a few varieties and by change of land use, also in the case of wild relatives due to habitat loss.

Banana germplasm resources are the important material base for new varieties breeding, biological technology, teaching, cientific development and their collection as well as the preservation in terms of in-depth research that enjoys very high value in the international sector. Some countries such as the Philippines, Vietnam, Indonesia and etc have put in place national genebanks for saving banana germplasm resources. Interestingly China is rich in wild banana germplasm resources, especially those containing cold, drought and disease-specific genes, which havegarnered more and more attention from banana growers, IPGRI and INIBAP organization alike. There are more than 210 domestic and foreign *Musa* germplasm in the already built banana germplasm nursery of our country. These germplasm provides a safe network for banana breeding. This is especially true of some germplasm which has specific traits that provide fast and convenient conditions for breeding new banana varieties. Hence, the importance of research on genetic diversity of banana germplasm resources is self-evident.

In a field survey, our team of researchers discovered a potential banana mutant strain (named as 'HD-1'). Years of observation revealed that it has a short stem, thick leaves, fine fruit and other good economic traits, and these traits can be genetically expressed and stably inherited. With the help of morphological taxonomy, cytology and molecular marker, this paper has identified the preliminary genetic relationship of 'HD-1'. It is common knowledge that Brazil is one of the most important domestic banana cultivar, so is Hainan province in China. Needless to say we use Brazil banana as a controlled variable to compare its resistance.Finally, we make a preliminary evaluation of the strain for subsequent experiments so as to ultimately approve early and reliable basis for the final approval of new varieties, to provide some ideas and to pave the way for banana breeding innovation.

Materials and Methods

Morphological classification of banana strain 'HD-1'

Treatment application and experimental design: By ways of using field grown banana strain 'HD-1' as an object and selecting 15 well-grown plants, we observe the bud period and the results of various morphological traits period.Simmonds classification believes that banana has two ancestors: M. *Acuminate Colla* (AA), and M. *Balbisiana Colla* (BB). Banana cultivators had evolved from two original banana through intraspecific or interspecific hybridization. According to the chromosome ploidy of all varieties and the similarity degree with two parents, the research team has used 15 kinds of major morphological characteristics to divide by scoring method. The characteristics and M. *Acuminate Colla* (AA) approximation is recorded as 1 point, and M. *Balbisiana Colla* (BB) approximation is recorded as 5 points. A transitional form between the two is recorded as 2 points, 3 points, 4 points respectively. Scores of the M. *Acuminate Colla* (AA) and M. *Balbisiana Colla* (BB) are 15 points and 75 points. Table 1 lists the banana traits used in the scoring system and banana is divided into 16 types based on scores and banana ploidy, which is shown in Table 2 (Simmonds *et al*., 1995a).

Table 1. The comparison of the properties between *Musa acuminata* Colla and *Musa balbisiana* colla.

Properties	*M. Acuminate* Colla (AA)	*M. Balbisiana* Colla (BB)
pseudostem color	Or deep or shallow, with brown or black spots	No significant or not
Petiole groove	The edge upright or outward, with membranous below, not tightly wrapped pseudostem	Edge down, the lower leaves no wings, wrap pseudostem
Fruit axis	Generally have pastel or fur	Smooth and glabrous
Stem	short	Long
Ovule	Each ventricle with two rows of neat ovule	Each ventricle with four lines of irregular ovule
Bracts shoulder	Generally higher(the high of bract stem to the most wide/the high of bract < 0.28)	Generally low and wide (the radio>0.30)
Bracts curling degree	Bracts bend outward and upward roll	Bracts only lifted, but not revolute
The formation of bracts	Lanceolate or long ovate	broadly ovate
Bracts cutting-edge	Acute	obtuse
Bract lustre	External red,dark purple or yellow, pink, dark purple or brown internal	Outside of the obvious brown-purple, interior is bright crimson
Bracts fade	Internal top to down gradually faded to yellow	Internal uniform fade
Bud scars	Projection	Micro-projection
Male flowers free flap shape	More or less wrinkles under the petal tip	Rare wrinkles
Male flowers are petal color	Milk white	More or less pink
Lustre of the stigma	Orange,brilliant yellow	Cream color, light yellow or pink

Table 2. The classification of edible banana.

Multiplicity of chromosome	Type of genome formula	Evaluation score	Combination (♀×♂)
3×	AAA	15-21	(AA) × AA
4×	AAAA	15-20	(AAA) × AA
2×	AA	16-33	AA×AA
3×	AAB	26-46	AABB×AA/(AB)×AA
4×	AAAB	27-53	(AAB)×AA/(AAB)×BB
4×	AABB	45-48	(ABB)×AA
2×	AB	46-48	BB×AA/(AB)×AA
3×	AAB	59-63	AABB×BB
4×	ABBB	63-67	(ABB)×BB
2×	BB	75	BB×BB

Karyotype analysis of banana strain 'HD-1'

Treatment application and experimental design: The tested materials are 'HD-1' plantlets with five leaves a heart.

Methods of chromosome flaking: Through technique of wall degradation (Braun *et al*., 1975), hypotonic treatment of making chromosomal preparations (Karami *et al.,* 2015), and then microscopic examination of the prepared slides after drying;researchers have found a good dispersion of medium-term cells at low magnification, observed and photograghed at high magnification. It is then followed by a selection of 30 well dispersed metaphase cells to count the total number of chromosomes. Karyotype analysis of the chromosome image is going with MATLAB toolbox after importing the image into computer (Dillon *et al.*, 2015).

Chromosome relative length is the ratio of the length of the chromosome and the total length of all chromosomes.

Arm ratio i.e., the ratio of the long arm and the short arm of the chromosome.

Based on arm ratio, with the provisions of two point four district system as standard, as shown in Table 3 (Sehlarbaum, 1984).

Relative length coefficient is the ratio of the length of chromosome and average length of all chromosomes. Based on classification method that Guo Xin-rong put forward in 1972, such as I, R, L≤0.75, is short chromosome(S); 0.76≤I, R, L≤1.00, is the medium-short chromosome(M1); 1.01≤I, R, L≤1.25, is the medium-long chromosome (M2); I, R, L≥1.26, is the long chromosome (L).

According to the length ratio and arm ratio of the chromosome, we establish the degrees of symmetry and asymmetry, and divide them into 12 types, as shown in Table 4.

Based on the characteristics and length of the chromosome, we pair the homologous chromosome. We arrange the chromosomes in a cell from big to small, the same length ones are based on arm ratio, the special markered ones (*e.g.*, along with satellite chromosome) however are for special arrangement.

Table 3. Chromosome classification criteria.

Centromere position	Arm ratio (L/S)
Median centromere (M)	1.00
Median centromere region (m)	1.01-1.70
Sub-median centromere region (sm)	1.71-3.00
Sub-telocentric centromere region (st)	3.01-7.00
Telocentric centromere region (t)	≥7.01
Telocentric centromere (T)	∞

ISSR molecular markers of banana strain 'HD-1'

Treatment application and experimental design: Varieties tested resources come from Chinese Academy of Tropical Agricultural Sciences (Table 5), researchers take the banana younger leaves that have yet to expand into the liquid nitrogen tank, immediately put it back to the lab and save the spare -80^{o}C low temperature conditions. Banana genomic DNA is extracted by improved CTAB method (Cota-Sánchez *et al.,* 2006), and used after quantitative detection and the concentration measurement of DNA. Next, we make genomic DNA as template in ISSR analysis, screen out the clear, stable and reproductive ones, and subsequently select more bands primers from 100 ones that the UBC has released, for the following experiments.

ISSR primers screening: With genomic DNA as template for ISSR analysis as shown in Table 6 in the 100 primers screened band, good stability and repeatability and relatively clear bands that have more primers are used for 19 banana germplasm genome DNA of ISSR-PCR amplification, for subsequent experiments.

ISSR-PCR reaction system and PCR amplification procedure: ISSR-PCR reaction system is 25 μL (Tikunov *et al*., 2003), including DNA template 20 ng, Taq enzyme 1.5 U, concentration of dNTPs 0.2 mmol/L, concentration of Mg^{2+} 2 mmol/L, primer concentration 0.4 μmol/L, sterile water 25 μL, operated on the ice with PCR instrument to amplify. Amplification procedures are as follows: 94^{o}C pre-denaturation 4 min; 94^{o}C denaturation 30 s, Tm 40 s, 72^{o}C extension 90 s, 35cycles; 72^{o}C extension 8min; finally conserve at 4^{o}C.

Table 4. By symmetric to asymmetric karyotype.

Max/Min	Percentage of chromosomes whose arm radio greater than 2			
	0.0	0.01—0.50	0.50—0.99	1.00
＜2:1	1A	2A	3A	4A
2:1—4:1	1B	2B	3B	4B
＞4:1	1C	2C	3C	4C

Note: 1A is the most symmetrical karyotype; 4C is the most asymmetrical karyotype

Table 5. Varieties and code of bananas

Code	Variety	Code	Variety
1	HD-1	11	Taiwan banana 2
2	Sanya dwarf banana	12	Mexico's banana
3	Baoting dwarf banana	13	Huanong 7
4	Honghe dwarf banana	14	Huanong sweet banana
5	Beida dwarf banana	15	Vallery
6	Diaoluo dwarf banana	16	Brazil
7	Jianfeng dwarf banana	17	Williams
8	Yitong dwarf banana	18	Truncate-tailed
9	Pubei dwarf banana	19	High foot mine
10	Tianbao dwarf banana		

With 2000 bp DNA Marker as a relative molecular mass standard, we mix 10 μL PCR products and 2 μL 6× Loading Buffer for electrophoretic separation about 1 hour, under 120 V electrophoresis constant pressure. After electrophoresis, photograph in a gel imaging system and save the image.

Every band of the electrophoretogram represents a primer binding site. According to the bands, we record all the binary data, with belt recorded as '1', and '0' for no belt. We calculate the percentage of all polymorphic loci based on the data obtained, analyze the data to calculate the similarity coefficient with NTSYS software, and UPGMA method for clusteringand establish the tree diagram.

Study on resistance of 'HD-1' banana strain seedling

Treatment application and experimental design: Brazil and 'HD-1' seedlings with five leaves are used as material derived from Chinese Academy of Tropical Agricultural Sciences. Brazil seedling height is 35-40 cm compared to 'HD-1' which is 30-35 cm, its robust growth with no plant diseases and insect pests.

Chilling resistance test of the 'HD-1' and Brazil banana seedlings: Two varieties of banana seedlings are divided into control group (CK) and treatment group, cultured for 24 hours at room temperature (25°C). The CK continues to cultivate at ambient temperatures, the treatment group is transferred to artificial climate box, and is subject to stress test. The condition of artificial climate box is at 7°C, light 300 μmol·m^{-2}·s^{-1}, relative humidity 85%-90%, cooling process starts from 25°C uniformly. After 24 hours stress test, we wash the second leaf of the seedling with 7°C distilled water, dry and then remove midrib and leaf margin for various physiological indexes measurement. Each treatment is repeated 3 times.

Drought resistance test of the 'HD-1' and Brazil banana seedlings: Seedlings are transformed into 1/2 Hoagland solution after roots have been washed. Water planting conditions are as follows: light 300 μmol·m^{-2}·s^{-1}, illumination time 14 h/d, day and night temperature 27°C /21°C, relative humidity 85%-90%, continued ventilation on nutrition solution with electric air pump during the culture period. After 3 days, we change the nutrient solution and add 10% PEG-6000 solution in 1/2 Hoagland for moderate stress treatment (Zimmer-Prados *et al*., 2014). After 24 hours, we wash the second leaf of the seedling with distilled water, dry and remove midrib and leaf margin for various physiological indexes measurement. Each treatment is repeated 3 times.

Salt resistance test of the 'HD-1' and Brazil banana seedlings: Seedlings are transformed into 1/2 Hoagland solution after roots being washed. Replace the nutrient solution every 3 days. Water planting conditions are the same as above. We set salt stress level after preculture for 3 days. Each species is divided into CK and treatment group.The CK remains the same, the final concentration of NaCl treatment group is 60 mmol/L in nutrient solution, then we investigate salt injury symptoms. Following the above procedure, we wash the second leaf of the seedling with distilled water, dry, remove midrib and leaf margin for various physiological indexes measurement. Each treatment is repeated 3 times.

***Fusarium* Wilt resistance test of the 'HD-1' and Brazil banana seedlings:** Test bacteria is *Fusarium oxysporum* f. sp. *cubense* race 4 (Foc 4), provided by the Biological Technology Research Institute of CATAS using root dipping inoculation method, with inoculum being 2×10^7 spores /mL suspension of conidia. We wash the root of banana seedlings and cut off part of the root tip. Treatment group is soaked in the spore suspension for 1 hour; the CK is soaked in sterile water, and then transplanted them back to the cup with 120°C high temperature sterilization of sterile soil. Each group has15 seedlings, and other culture conditions are kept constant.

Table 6. Nucleotide sequence of the primer used in the ISSR experiment.

Primers number	Sequence (5′-3′)	Primers number	Sequence (5′-3′)
801	ATA TAT ATA TAT ATA TT	857	ACA CAC ACA CAC ACA CYG
802	ATA TAT ATA TAT ATA TG	858	TGT GTG TGT GTG TGT GRT
803	ATA TAT ATA TAT ATA TC	859	TGT GTG TGT GTG TGT GRC
804	TAT ATA TAT ATA TAT AA	860	TGT GTG TGT GTG TGT GRA
805	TAT ATA TAT ATA TAT AC	861	ACC ACC ACC ACC ACC ACC
806	TAT ATA TAT ATA TAT AG	862	AGC AGC AGC AGC AGC AGC
807	AGA GAG AGA GAG AGA GT	863	AGT AGT AGT AGT AGT AGT
808	AGA GAG AGA GAG AGA GC	864	ATG ATG ATG ATG ATG ATG
809	AGA GAG AGA GAG AGA GG	865	CCG CCG CCG CCG CCG CCG
810	GAG AGA GAG AGA GAG AT	866	CTC CTC CTC CTC CTC CTC
811	GAG AGA GAG AGA GAG AC	867	GGC GGC GGC GGC GGC GGC
812	GAG AGA GAG AGA GAG AA	868	GAA GAA GAA GAA GAA GAA
813	CTC TCT CTC TCT CTC TT	869	GTT GTT GTT GTT GTT GTT
814	CTC TCT CTC TCT CTC TA	870	TGC TGC TGC TGC TGC TGC
815	CTC TCT CTC TCT CTC TG	871	TAT TAT TAT TAT TAT TAT
816	CAC ACA CAC ACA CAC AT	872	GAT AGA TAG ATA GAT A
817	CAC ACA CAC ACA CAC AA	873	GAC AGA CAG ACA GAC A
818	CAC ACA CAC ACA CAC AG	874	CCC TCC CTC CCT CCC T
819	GTG TGT GTG TGT GTG TA	875	CTA GCT AGC TAG CTA G
820	GTG TGT GTG TGT GTG TC	876	GAT AGA TAG ACA GAC A
821	GTG TGT GTG TGT GTG TT	877	TGC ATG CAT GCA TGC A
822	TCT CTC TCT CTC TCT CA	878	GGA TGG ATG GAT GGA T
823	TCT CTC TCT CTC TCT CC	879	CTT CAC TTC ACT TCA
824	TCT CTC TCT CTC TCT CG	880	GGA GAG GAG AGG AGA
825	ACA CAC ACA CAC ACA CT	881	GGG TGG GGT GGG GTG
826	ACA CAC ACA CAC ACA CC	882	VBV ATA TAT ATA TAT AT
827	ACA CAC ACA CAC ACA CG	883	BVB TAT ATA TAT ATA TA
828	TGT GTG TGT GTG TGT GA	884	HBH AGA GAG AGA GAG AG
829	TGT GTG TGT GTG TGT GC	885	BHB GAG AGA GAG AGA GA
830	TGT GTG TGT GTG TGT GG	886	VDV CTC TCT CTC TCT CT
831	ATA TAT ATA TAT ATA TYA	887	DVD TCT CTC TCT CTC TC
832	ATA TAT ATA TAT ATA TYC	888	BDB CAC ACA CAC ACA CA
833	ATA TAT ATA TAT ATA TYG	889	DBD ACA CAC ACA CAC AC
834	AGA GAG AGA GAG AGA GYT	890	VHV GTG TGT GTG TGT GT
835	AGA GAG AGA GAG AGA GYC	891	HVH TGT GTG TGT GTG TG
836	AGA GAG AGA GAG AGA GYA	892	TAGATCTGATATCTGAATTCC C
837	TAT ATA TAT ATA TA ART	893	NNN NNN NNN NNN NNN
838	TAT ATA TAT ATA TA ARC	894	TGGTAGCTCTTGATCANN NNN
839	TAT ATA TAT ATA TA ARG	895	AGAGTTGGT AGC TCT TGA TC
840	GAG AGA GAG AGA GAG AYT	896	AGGTCGCGGCCGCNNNNNNATG
841	GAG AGA GAG AGA GAG AYC	897	CCGACTCGAGNNNNNNATGTGG
842	GAG AGA GAG AGA GAG AYG	898	GATCAAGCTTNNNNNNATGTG G
843	CTC TCT CTC TCT CTC TRA	899	CATGGTGTTGGTCATTGT TCC A
844	CTC TCT CTC TCT CTC TRC	900	ACTTCCCCA CAG GTT AAC ACA
845	CTC TCT CTC TCT CTC TRG	Degenerate primers bases code is as follows:	
846	CAC ACA CAC ACA CAC ART	N=（A,G,C,T）	
847	CAC ACA CAC ACA CAC ARC	R=(A,G)	
848	CAC ACA CAC ACA CAC ARG	Y=(C,T)	
849	GTG TGT GTG TGT GTG TYA	B=(C,G,T) (i.e. not A)	
850	GTG TGT GTG TGT GTG TYC	D=(A,G,T) (i.e. not C)	
851	GTG TGT GTG TGT GTG TYG	H=(A,C,T) (i.e. not G)	
852	TCT CTC TCT CTC TCT CRA	V=(A,C,G) (i.e. not T)	
853	TCT CTC TCT CTC TCT CRT	K=(G,T) (Keto in large groove)	
854	TCT CTC TCT CTC TCT CRG	M=(A,C) (aMino in large groove)	
855	ACA CAC ACA CAC ACA CYT	S=(G,C) (Strong [3 H-bonds])	
856	ACA CAC ACA CAC ACA CYA	W=(A,T) (Weak [2 H-bonds])	

Determination method of physiological index of leaves: The cell membrane permeability is measured by conductivity meter method (Matuszewska *et al*., 1984), Chlorophyll is extracted with the method of acetone ethanol mixture(Shi *et al*., 2016), soluble protein content is measured brilliantly with (not too sure but I assume this is what you to say) the Blue G-250 method (Snyder *et al.*, 1978).Malondialdehyde (MDA) content is resolved with thiobarbituric acid (TBA) colorimetry, and superoxide dismutase (SOD) activity is determined by nitro blue tetrazolium (NBT) reduction method.Proline content is measured by acidic indene three colorimetric, soluble sugar content is measured by anthrone colorimetry (MU *et al.,* 2010).

Banana *Fusarium* wilt resistance evaluation standard: Banana seedling wilt disease classification standard includes internal and external symptoms (Xie *et al*., 2009), Table 7. After inoculation, we analyze external symptoms of banana seedlings in 14 d, 28 d, 35 d respectively, and look into their internal symptoms in 35 d. External symptom survey site is its blade, internal site is its bulb, slit bulb and statistic bulb discoloration occurs. Survey data of 35 d are used as resistance evaluation premise. Suspected diseased plants are possibly caused by other pests and diseases have not entered into the survey results.

Convert the incidence grade of the banana strain to disease severity. Disease severity data retain 1 decimal place. The calculation formula is as follows:

$$\text{Disease severity} = \frac{\sum (\text{Level value x Number of trees at the level})}{\text{Number of investigation}}$$

First resistance level of the strains is determined by way of external and internal symptoms respectively: the resistance evaluation criteria (Table 8). We synthesize internal and external symptoms resistance level and obtain resistance level of the strain. Confirmed criteria: resistance level of a strain tends to be susceptible to the disease between internal and external symptoms. For example, if external symptoms show high resistance, while internal symptoms show moderate resistance, then the strain is moderate resistance (Liu *et al*., 2008).

Data processing: Using ANOVA process of SAS9.2 software for processing differences significant test, Duncan's new multiple range method for multiple comparisons between means.

Results

Morphological classification of banana strain 'HD-1'

Using Simmonds taxonomy (1995a), 15 characters of the banana strain have been observed, we then describe and rate it accordingly. Table 9 shows that the total score of the banana strain 'HD-1' is 21 points. According the Table 2, we know that the ploidy is 3×, type AAA.

Karyotype analysis: The test records chromosome numbers of 30 well dispersed metaphase cell and finds that the chromosome number of the banana strain'HD-1'is 33 (Fig. 1), the ideogram is shown in Fig. 2. As showed in Table 10, the karyotype fomula is 2n=3x=33=2 L +3 M2+4 M1+2 S, and No.1,2,3 chromosomes are submetacentric chromosomes, the rest are metacentric chromosomes, with no satellite chromosomes found. The maximum of chromosome relative length is 13.428%, the minimum is 5.428%; the variation of relative length coefficient is between 0.597-1.478; the length ratio of the longest chromosome and the shortest chromosome is 2.476.Arm ratios are less than 2, in the range of 1.063 - 1.846; classified as'1B'type, karyotype is symmetrical.

ISSR molecular markers of banana strain 'HD-1'

Banana genomic DNA extraction results: With improved CTAB method, DNA samples prepared are assayed by using ultraviolet spectrophotometer and 1% agrose gel electrophoresis. The ratios of A_(260)/A_(280) are 1.81-1.90. DNA bands of the 1% agrose gel electrophoresis are clear, regular, and tailless (Fig. 3).

Results of screening and amplification of banana ISSR primers: Table. 11 is an indicator of the result of genomic DNA amplification of ISSR-PCR of 19 banana varieties and screened 6 primer sequences (Fig. 4). 41 bands have been tested from the final 6 ISSR primers of DNA. Of which 34 are of polymorphism, accounting for a total of 82.9%, the amplified DNA fragments are between 200-2500 bp. Data shows that the genetic diversity of banana varieties are very rich at the molecular level. The amplification results of 813, 818, 868 primers are shown in Figs. 5-7.

Clustering analysis of 19 banana germplasm: Similar coefficients are calculated on 6 primers amplified data by using NTSYS statistical analysis software. We build a tree clustering analysis graph of genetic relationship between varieties (Fig. 8). According to the clustering tree diagram, the similarity coefficients of 19 banana germplasm are in the range of 0.49-0.94. The result of clustering analysis shows that: 19 varieties can be divided into 3 groups when the genetic distance at the entropy value is 0.62. The first group includes varieties of 'HD-1', Pubei dwarf banana, Tianbao dwarf banana, Yitong dwarf banana, Mexico banana, vallery; the second group includes varieties of Sanya dwarf banana, Qi Wei, Baoting dwarf banana, Honghe dwarf banana, Beida dwarf banana, *etc.*; the third group includes varieties of Brazil, Williams, and High foot mine. 'HD-1', Pu Bei dwarf banana and Tianbao dwarf banana are grouped as one category, together with Yitong dwarf banana, Mexico banana and vallery which are categorized as one class. 'HD-1' and Brazil have the smallest similarity coefficient: 0.31, whereas 'HD-1', Pubei dwarf banana, and Tianbao dwarf banana have the largest similarity coefficient: 0.81. Thus, 'HD-1' and Pubei, Tianbao dwarf banana benefits from the closest relationship.

Table 7. Grades of Fusarium wilt disease at seedling stage.

Disease grade	Symptom	
	Internal symptoms	Morphological symptom
1	Bulbs don't change color	Leaves don't turn yellow,health
2	Bulbs don't change color, but in root and bulb junction discoloration	Lower leaves slight yellowing
3	0-5% bulb discoloration	Most of the lower leaves yellowing, upper leaves begin to change color
4	6%-20% bulb discoloration	Most or all of the leaves yellowing
5	21%- 50% bulb discoloration	Dead plants
6	More than 50% bulb discoloration	
7	All bulbs discoloration	
8	Dead plants	

Table 8. Integrated evaluation standard for Fusarium wilt at seedling stage.

Resistance grade	Severity grade		Resistance level
	Morpholofical symptoms	Internal symptoms	
1	≤1.0	≤1.0	High resistance (HR)
2	1.1—2.0	1.1—3.0	Moderate resistance (MR)
3	2.1—3.0	3.1—5.0	Susceptibility (S)
4	3.1—4.0	5.1—8.0	High susceptibitility (HS)

Table 9. Traits and scores of banana strain 'HD-1'.

Character	'HD-1'	Score
Pseudostem color	Light purplish red	1
Petiole groove	The edge upright and outward, not tightly wrapped pseudostem	1
Fruit axis	A lot of short fur	1
Stem	Shorter	2
Ovule	Each ventricle with three rows of ovule	3
Bracts shoulder	High	1
Bracts curling degree	Bracts revolute	1
The formation of bracts	Lanceolate	1
Bracts cutting-edge	Sharp circle	2
Bract lustre	The exterior is red brown, internal is red	1
Bracts fade	From top to bottom fade	1
Bud scars	Projection	1
Male flowers free flap shape	Wrinkles under the petal tip	1
Male flowers are petal color	Creamy yellow	3
Lustre of the stigma	Orange	1
Scores		**21**

Table 10. Relative length, arm ratio and type of the chromosome in 'HD-1' banana strain.

Number	Relative length (%) (S + L = T)			Relative length coefficient	Arm ratio (L/S)	centromere classification	Type
1.	4.857	8.571	13.428	1.478	1.765	sm	L
2.	4.286	7.429	11.715	1.289	1.733	sm	L
3.	3.714	6.857	10.571	1.163	1.846	sm	M2
4.	4.857	5.429	10.286	1.131	1.118	m	M2
5.	4.571	4.857	9.428	1.037	1.063	m	M2
6.	3.429	5.429	8.858	0.974	1.583	m	M1
7.	3.714	4.857	8.571	0.943	1.308	m	M1
8.	4.000	4.286	8.286	0.911	1.071	m	M1
9.	3.429	3.714	7.143	0.786	1.083	m	M1
10.	2.857	3.429	6.286	0.691	1.200	m	S
11.	2.571	2.857	5.428	0.597	1.111	m	S

Note: sm is submedian region; m is median region

Table 11. Banana 19 germplasm primer amplification ISSR characteristics.

Primer code	(5′—3′) Primer equence	Number of bands	Number of polymorphic bands	Percentage of polymorphic	Annealing temperature
813	(CT)8T	4	3	75.0	52.2
818	C(AC)7AG	7	6	85.7	52.2
836	(AG)8YA	7	6	85.7	52.2
855	(AC)8YT	7	6	85.7	55.0
864	(ATG)6	7	5	71.4	53.0
868	(GAA)6	9	8	88.9	53.0
Total	—	41	34	82.9	—
Average	—	6	5	83.3	—

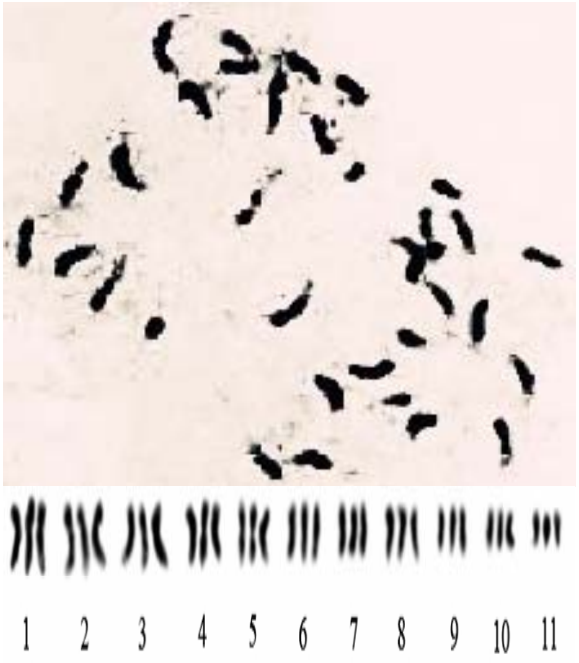


Fig. 1. Form of the Chromosome in ‘HD-1’ banana strain.
Note: A meansthe chromosome mitotic metaphase. B mean karyotypes diagram.
(Scale bar=5×10^{-6}m)

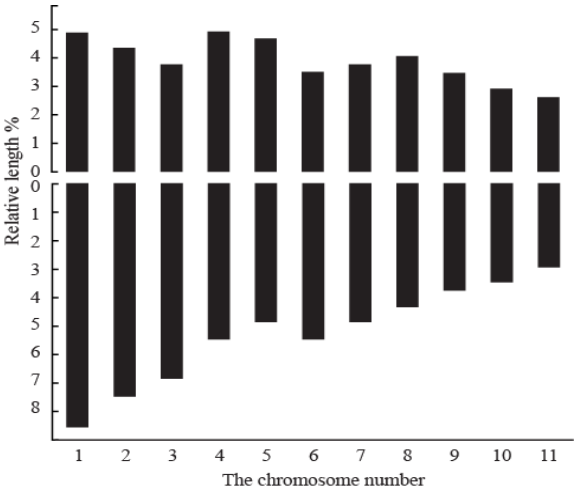


Fig. 2. Ideogram of ‘HD-1’ banana strain.

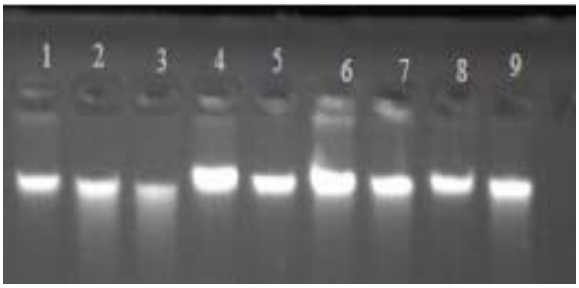


Fig. 3. Part of the DNA electrophoresis figure.

Resistance research of ‘HD-1’ banana seedling

Cold resistance comparison of the‘HD-1’and Brazil

1. The morphology of seedling leaves: As observation suggests, banana tends to show some apparent symptoms after chilling injury, such as cold spot or wilting margin of leaf, and even death, which is easy to detect and can be treated as the basic indicators of chilling injury. For the treatment group, when the interior leaves of Brazil seedlings are the wilting margin, the expanded leaves are all wilting; but when the interior leaves of HD-1 seedlings are normal, the expanded leaves are half wilting.

2. Effect of low temperature stress on relative conductivity and MDA content of Brazil and ‘HD-1’ banana leaves: As shown in Fig. 9, after 24hour of low temperature, their relative conductivity increases significantly, which indicates that the cell membrane was destroyed due to low temperature; the membrane permeability becomes larger. The relative conductivity of Brazil and ‘HD-1’ stress group each increases by 2.58 times and 2.38 times than the CK respectively. This suggests the Brazil banana is higher than ‘HD-1’, which indicates that the damage of their membrane systems are more consequential.

The MDA content of both banana strains increases significantly. Compared with the CK, the MDA content of the stress group of Brazil and ‘HD-1’ have increased by 45.3% and 40.8% respectively. And MDA is the product of the plant cell membrane lipid peroxide, so the cell membrane peroxidation level of Brazil is higher than ‘HD -1’.

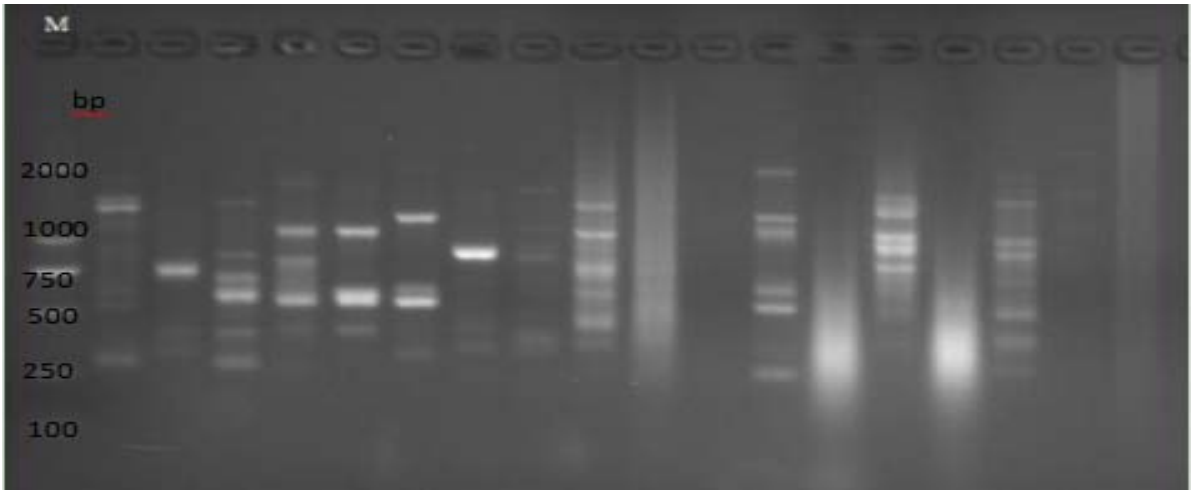


Fig. 4. Part of the primer on ISSR-PCR amplification results.
Notes: M, DL2000TM DNA Marker; 1-18 bands represented primers are 853-870(Table 6)

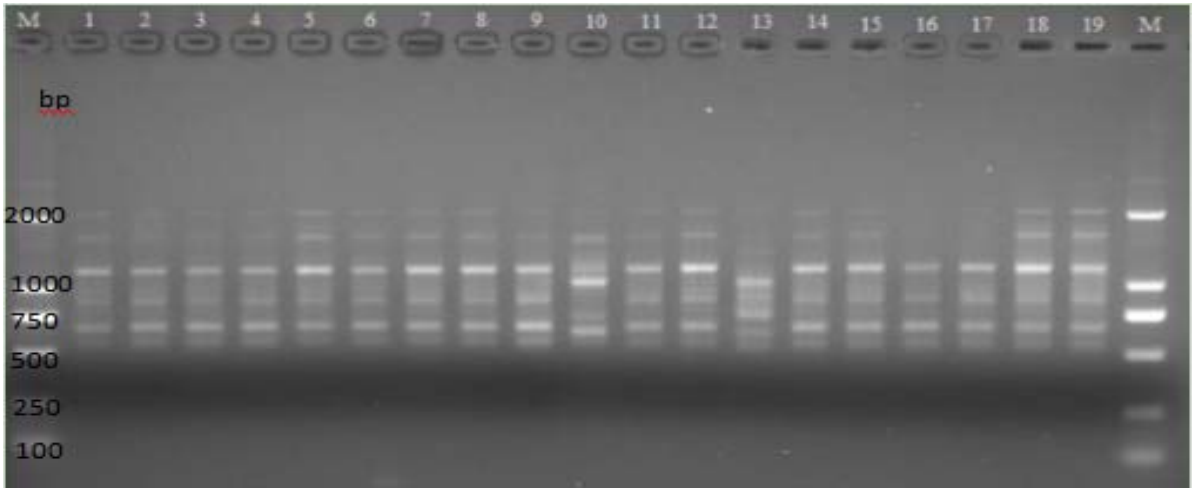


Fig. 5. PCR amplification electrophoretogram of primer 813 for 19 banana germplasm.
Note: M, DL2000TM DNA Marker; 1-19 bands represented varieties are shown in Table 5

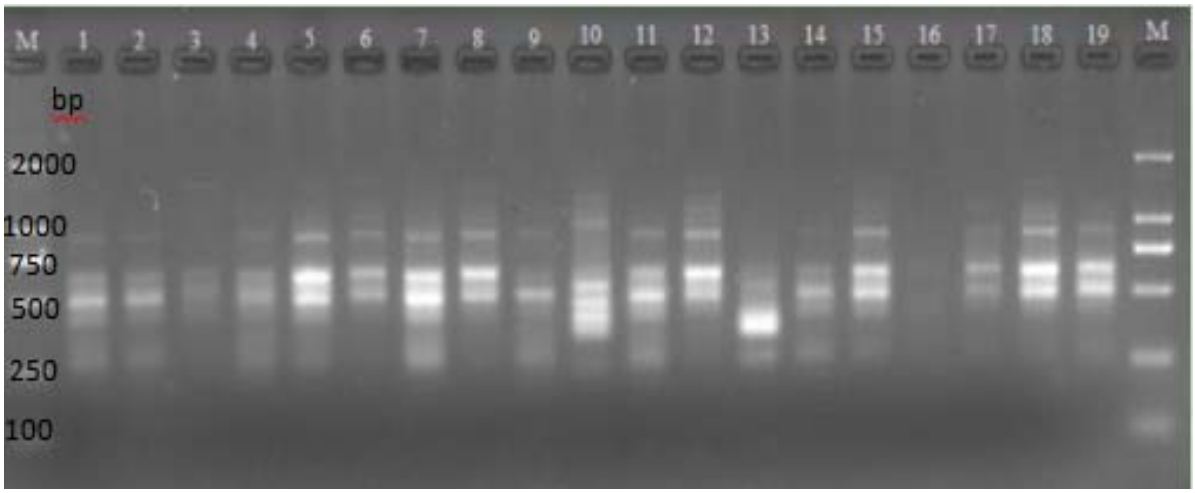


Fig. 6. PCR amplification electrophoretogram of primer 818 for 19 banana germplasm.
Note: M, DL2000TM DNA Marker; 1-19 bands represented varieties are shown in Table 5

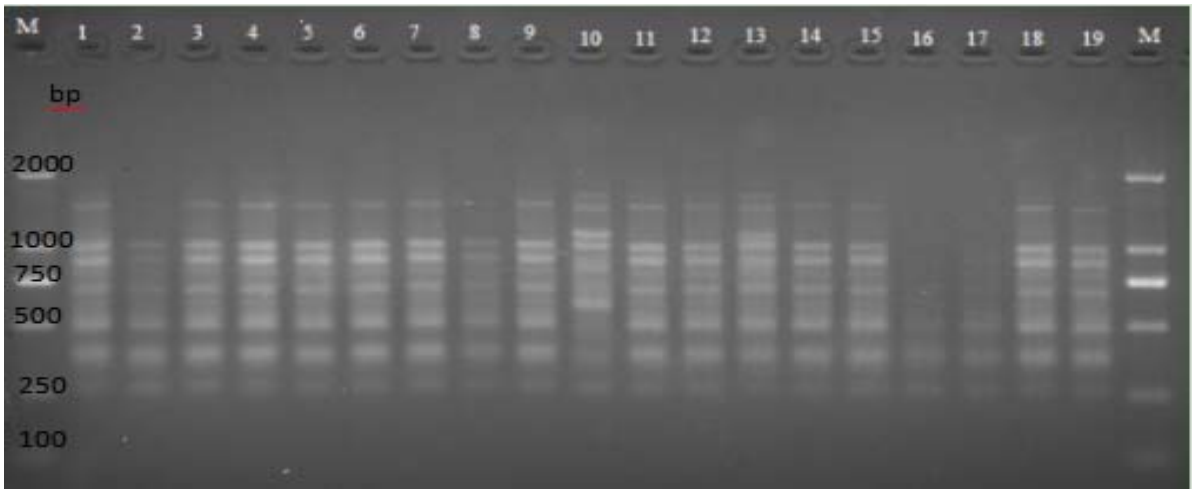
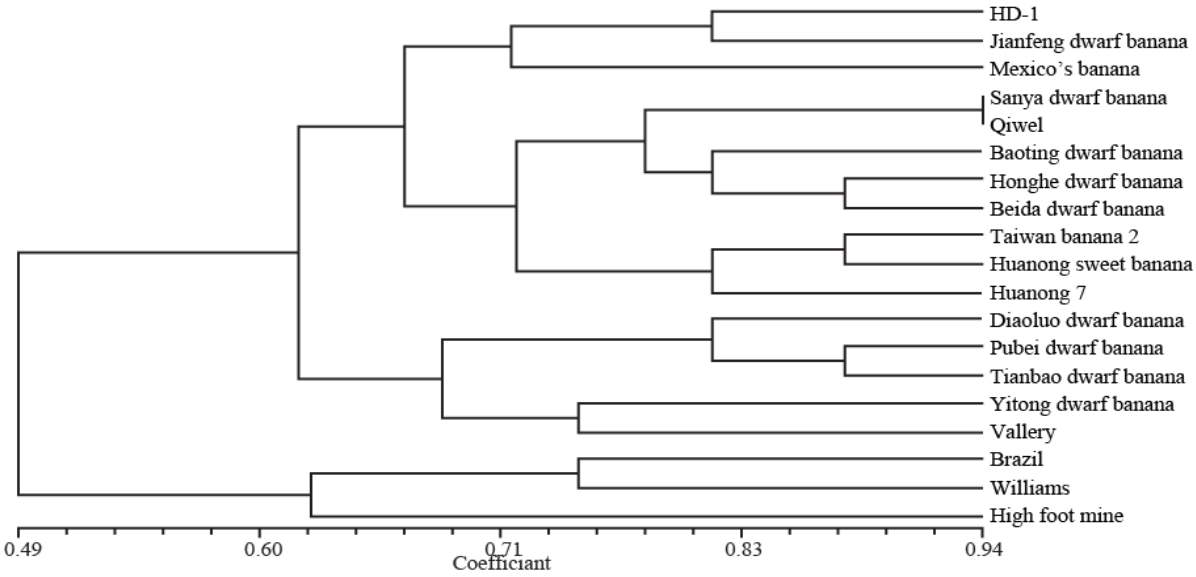


Fig. 7. PCR amplification electrophoretogram of primer 868 for 19 banana germplasm.
Note: M, DL2000TM DNA Marker; 1-19 bands represented varieties are shown in Table 5



Genetic similarities coefficient
Fig. 8. UPGMA dendrogram of 19 banana germplasm based on genetic similarity coefficient.

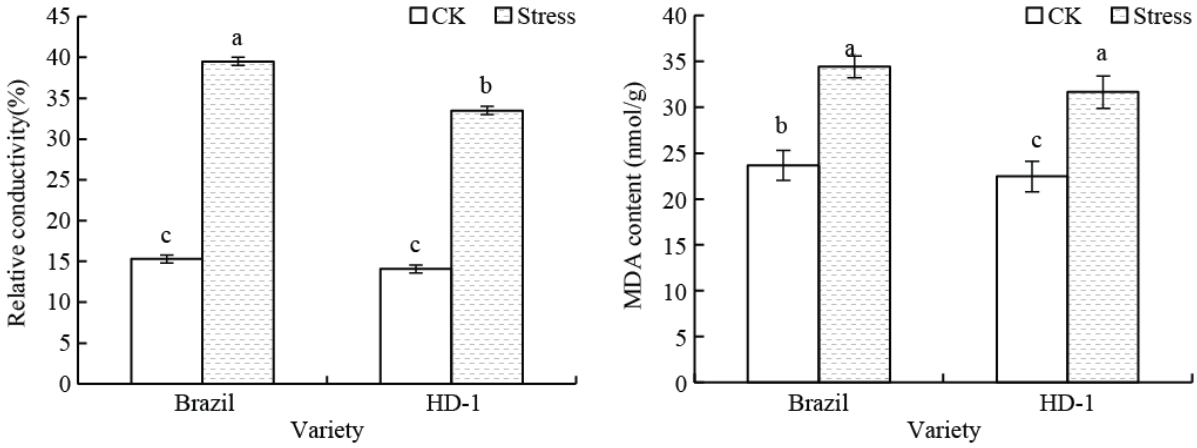


Fig. 9. Effect of low temperature stress on relative conductivity and MDA content of Brazil and 'HD-1' banana leaves.

3. Effect of low temperature stress on Chlorophyll content of Brazil and 'HD-1' banana leaves: As can be seen from Fig. 10, under low temperature stress, the total chlorophyll content of banana seedlings decreased notably than normal control. In comparison with the CK, the total chlorophyll content of the stress group of Brazil and 'HD-1'have decreased by 80.6% and 43.7% in turn. After the stress, the chlorophyll content of the 'HD-1'strain are found to be significantly higher than Brazil.

4. Effect of low temperature stress on leaf protective enzyme activity of Brazil and 'HD-1' banana seedlings: As shown in Fig. 11, after 24-hour 7^{o}C stress, the SOD and POD activities of the banana leaves have decreased remarkably. Compared with the CK, the SOD and POD activities of Brazil treatment group have declined by 49.4% and 53.5% respectively, with the 'HD-1' strain showing 22.8% and 38.4% reduction as the result. However, after low temperature stress, the SOD and POD activities of 'HD-1' banana strain seedling leaf are significantly higher than that of Brazil banana.

5. Effect of low temperature stress on the leaf osmotic adjusting substance of Brazil and 'HD-1' banana seedlings: From Fig. 12, after 24 h 7^{o}C osmotic stress, the osmotic regulation substances of banana seedlings leaf such as proline, soluble sugar, and soluble protein content have shown an notable increase.(try to swap the word "significantly" with a different word, there's too much repetition of this word) Compared with the CK, the treated group of Brazil banana has risen by 1.44 times, 3.60 times, and 1.08 times in turn; (again the word 'respectively' is used too many times) whilst the 'HD-1' banana strain increased by 1.47 times, 4.02 times, and 1.27 times. As a result, the osmotic adjustment substances of the banana seedlings leaf accumulate spontaneously when they suffer from low temperature, which enhances the ability of osmotic adjustment and maintenance ability of moisture, and the increase degree of 'HD-1' was more obvious than that of Brazil banana.

Drought resistance comparison of the 'HD-1' and Brazil

1. The morphology of seedling leaves: Through observation it is clear that if suffered from drought damage, the seedling leaves of Brazil banana treated group wilt, vein enation forms, leaf blade base has brown spots;and 'HD-1' treatment group seedling leaves have slightly water loss, and leaf margin are curling slightly.

2. Effect on the total chlorophyll content of 'HD -1' and Brazil banana under drought stress: As can be seen from Fig. 13, under drought stress, the total chlorophyll content of banana seedlings decrease than under normal control. Compared with the CK, the total chlorophyll content of the stress group of Brazil and 'HD-1'have reduced by 12.3% and 10.0%. The decline in chlorophyll content, to some extent, reflects the depletion in photosynthesis.

3. Effect on relative conductivity and MDA content of 'HD -1' and Brazil banana under drought stress: As shown in Fig. 14, the relative conductivity and MDA content of both banana strains increase after being treated with 10% PEG-6000 for 1d. Compared with the CK, the stress group of Brazil banana has a growth of 51.5% and 30.7%; and the 'HD-1' has increased by 34% and 21.1%. And MDA is the product of the plant cell membrane lipid peroxide, so the cell membrane peroxidation level of Brazil is higher than 'HD -1', which indicates that under the same processing conditions, Brazil banana seedlings membrane systems' suffers more damage than 'HD – 1', membrane lipid at a higher level.

4. Effect of drought stress on leaf protective enzyme activity of Brazil and 'HD-1' banana: From Fig. 15 it is shown that the SOD and POD activity of the banana leaves increase after drought stress. Compared with the CK, the SOD and POD activities of Brazil treatment group have risen by 1.50 times and 1.68 times; with the 'HD-1' strain showing a 1.6 times and 2.0 times increase. However, after low temperature stress, the SOD and POD activities of 'HD-1' banana strain seedling leaf are higher than that of Brazil banana. The expansion of Brazil banana is less than 'HD-1's'.

5. Effect of drought stress on the leaf osmotic adjusting substance and RWC of Brazil and 'HD-1' banana: From Fig. 16(a) and 16 (b) it is seen that the leaves of both banana strains would produce large amounts of proline, soluble sugar, and soluble protein after drought stress. Weigning against CK, the treated of Brazil banana has grown by 2.08 times, 1.64 times, and 1.91 times; the 'HD-1' banana strain increased by 2.46 times, 2.23 times, and 1.7 times. As a result, the osmotic adjustment substances of the banana seedlings leaf accumulate spontaneously when struck by low temperature, enhance the ability of osmotic adjustment and maintenance ability of moisture, and the inflated degree of 'HD-1' is more obvious than that of Brazil banana. Under moderate drought stress, the growth of proline and soluble sugar content in 'HD-1'leaves are larger than Brazil, but the soluble protein content is oppostie in contrast.

Under drought stress, the plant exhibites a decrease in relative water content. As shown in Fig. 17, the relative water content of banana leaves reduce after drought stressed for 24 hours. Compared with the CK, the treated group of 'HD-1' and Brazil banana have shrank by 12.4% and 16.2%, and 'HD-1' banana leaf water content is higher than Brazil banana's.

Salt resistance comparison of the'HD-1'and Brazil

1. The morphology of seedling leaves: As can be seen through the observation, treated with 60 mmol/L NaCl for 3 d, the seedling leaves of Brazil banana treated group wilt; part of the tip and leaf margin becomes withered and scorched, the leaf blade base shows brown spots; The 'HD-1' treatment group seedling leaves have slight water loss, with leaf margins curling slightly.

2. Effect of salt stress on Chlorophyll content of Brazil and 'HD-1' banana leaves: Seen from Fig. 18 it reveals that the total chlorophyll content of banana seedlings decrease than normal control after salt stress. Compared with the CK, the treated group of Brazil and 'HD-1' has decreased by 35.1% and 5.3 %. Chlorophyll content of 'HD-1' is still higher than that of Brazil banana.

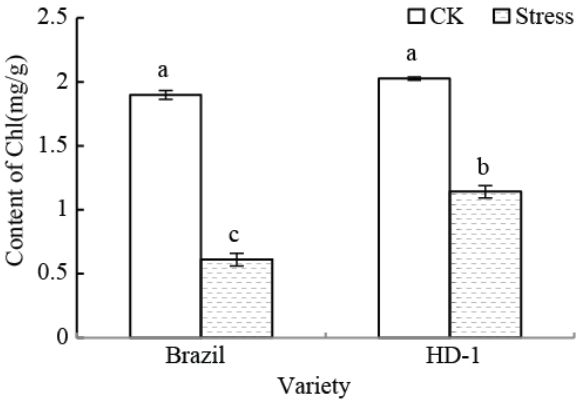


Fig. 10. Effect of low temperature stress on Chlorophyll content of Brazil and 'HD-1' banana leaves.

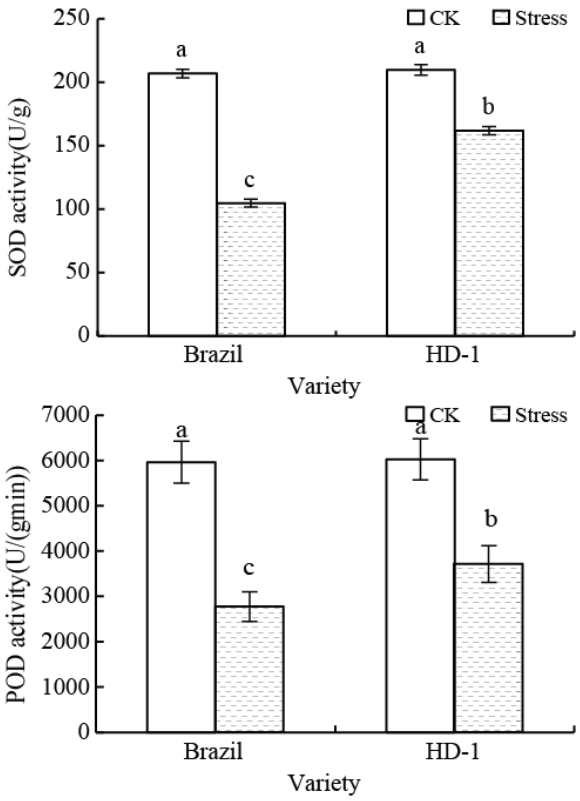


Fig. 11. Effect of low temperature stress on SOD and POD activity of Brazil and 'HD-1' banana leaves.

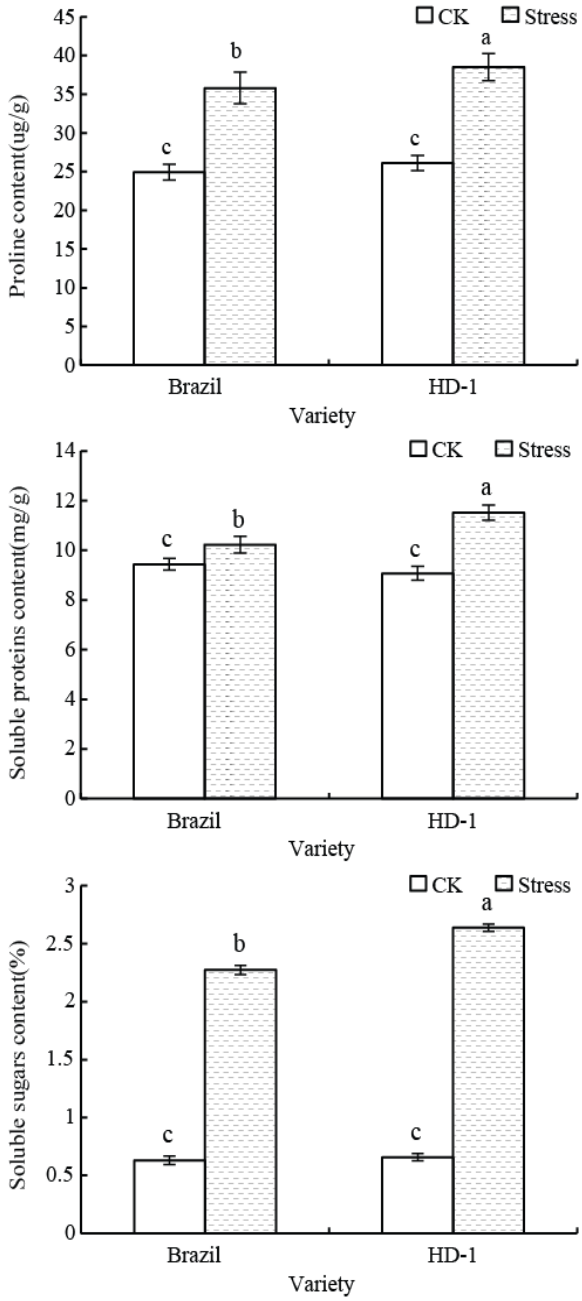


Fig. 12. Effect of low temperature stress on osmotic adjustment substance of Brazil and 'HD-1'banana leaves.

4. Effect of salt stress on leaf protective enzyme activity of Brazil and 'HD-1' banana: According to Fig. 20, the SOD and POD activities of the banana leaves increase after salt stress. Compared with the CK, the SOD and POD activities of Brazil treatment group have increased by 2.19 times and 1.91 times; 'HD-1' strain has increased by 2.49 times and 2.0 times. However, the increase of Brazil banana is smaller than 'HD-1'.

5. Effect of salt stress on the leaf osmotic adjusting substance and RWC of Brazil and 'HD-1' banana: Fig. 21(a) and 21(b) indicate that the leaves of both banana strains produce large amount of proline, soluble sugar, and soluble protein after salt stress. Compared with the CK, the treated Brazil banana has increased by 1.61times, 1.32 times, and 1.33 times; the 'HD-1' banana strain increased by 1.72 times, 1.69 times, 1.46 times. The increase of proline, soluble sugar and soluble protein content of 'HD-1' is bigger than Brazil banana.

As shown in Fig. 22 the relative water content of banana leaves reduce after salt stress for 3 d. Compared with the CK, the treated group of 'HD-1' and Brazil banana have decreased by 5.4% and 7.2%, and 'HD-1' banana leaf water content is higher than Brazil banana.

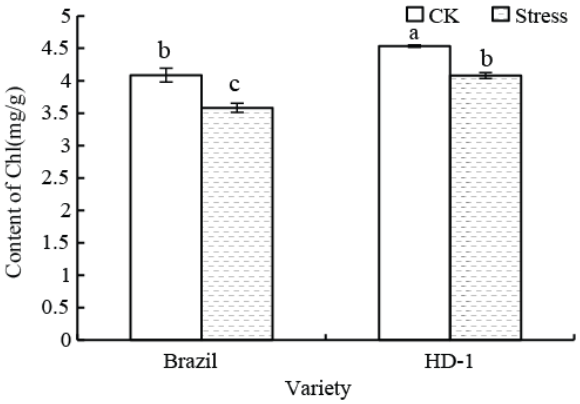


Fig. 13. Effect of drought stress on Chlorophyll content of Brazil and 'HD-1' banana leaves.

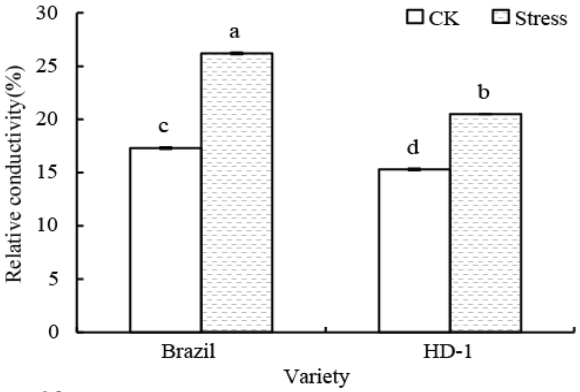


Fig. 14. Effect of drought stress on relative conductivity and MDA content of Brazil and 'HD-1' banana leaves.

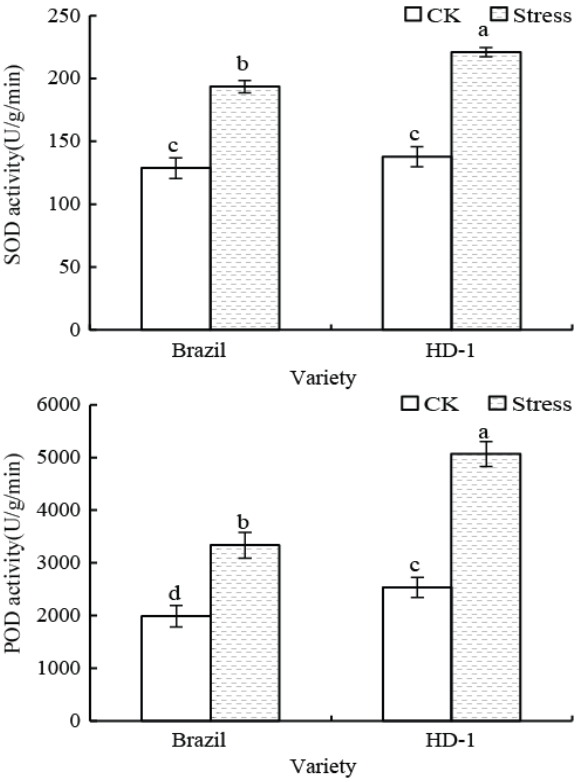


Fig. 15. Effect of drought stress on SOD and POD activity of Brazil and 'HD-1' banana leaves.

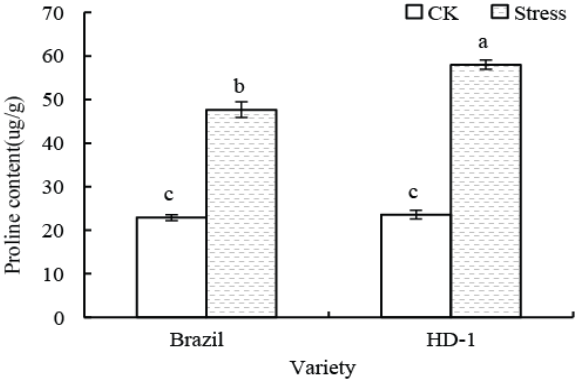


Fig. 16(a). Effect of drought stress on proline content of Brazil and 'HD-1' banana leaves.

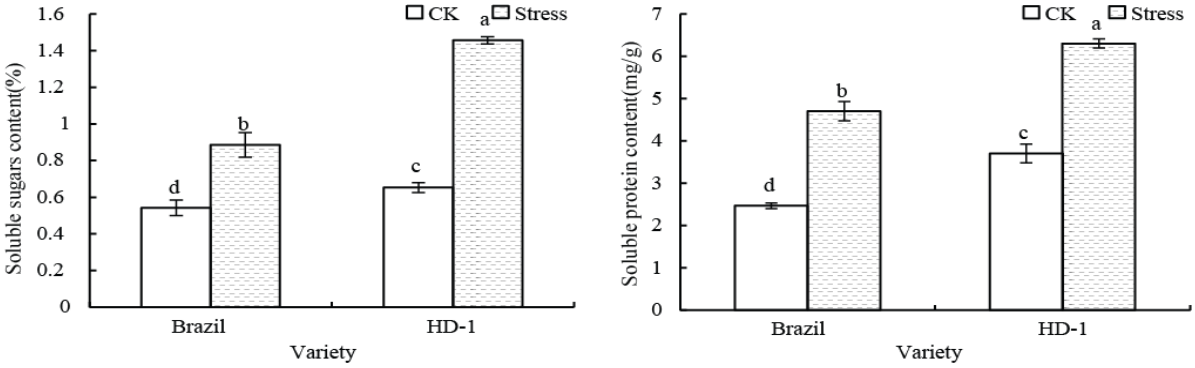


Fig. 16(b). Effect of drought stress on soluble sugars content and soluble proteins content of Brazil and 'HD-1' banana leaves.

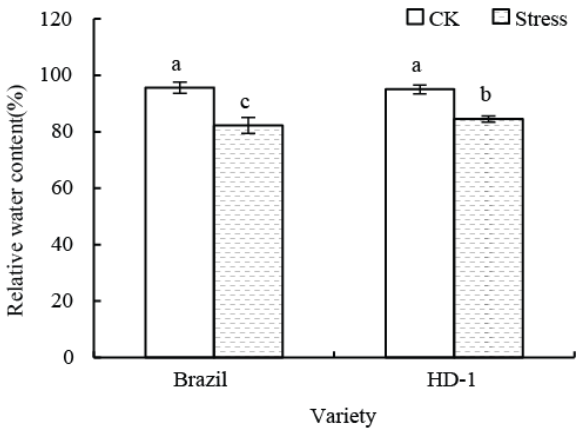


Fig. 17. Effect of drought stress on relative water content of Brazil and 'HD-1' banana leaves.

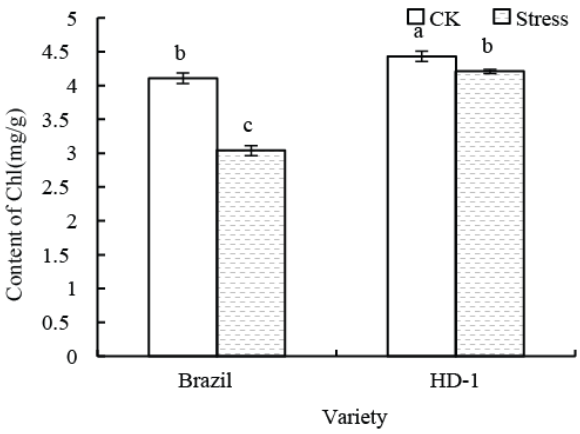
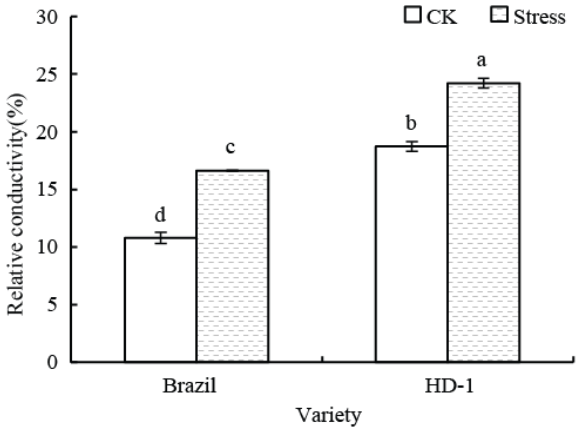


Fig. 18. Effect of salt stress on Chlorophyll content of Brazil and 'HD-1' banana leaves.



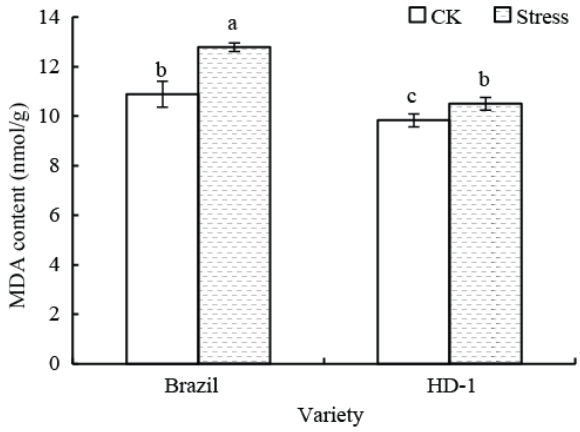
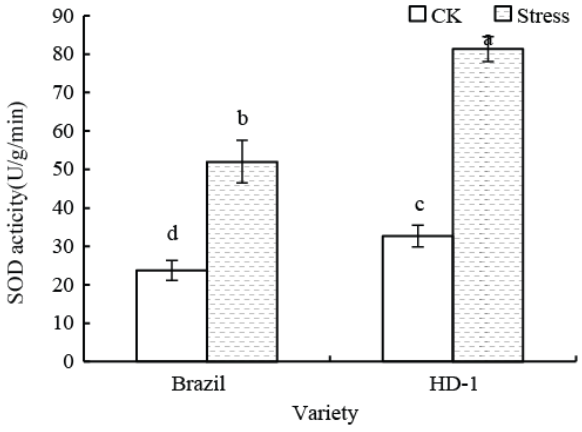


Fig. 19. Effect of salt stress on relatively activity and MDA content of Brazil and 'HD-1' banana leaves.



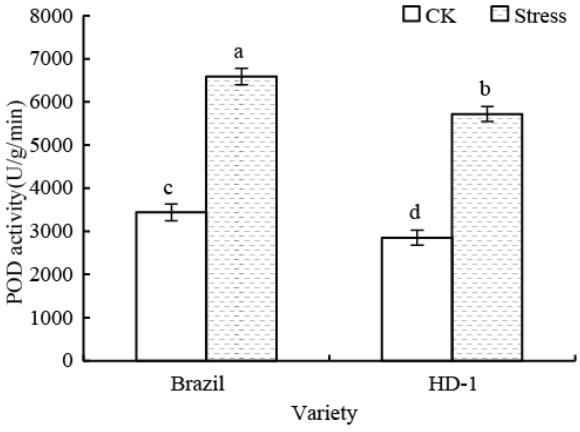


Fig. 20. Effect of salt stress on SOD and POD activity of Brazil and 'HD-1' banana leaves.

Table 12. The resistant evaluation results of two banana varieties.

Variety	External symptom		Inner symptom		Evaluation	
	Severity	Grade	Severity	Grade	Resistance grade	Resistance level
Brazil	3.3	4	5.2	4	4	HS
'HD-1'	2.6	3	4.1	3	3	S

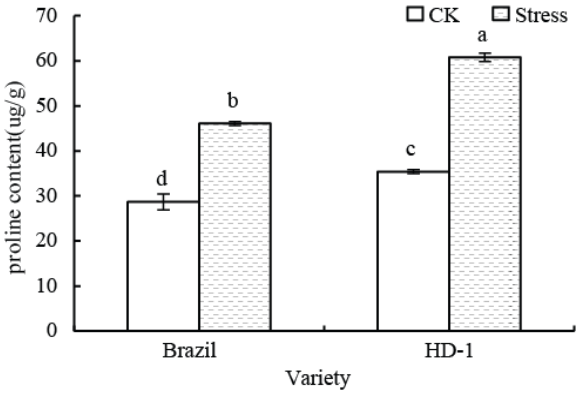


Fig. 21(a). Effect of salt stress on proline content of Brazil and 'HD-1' banana leaves.

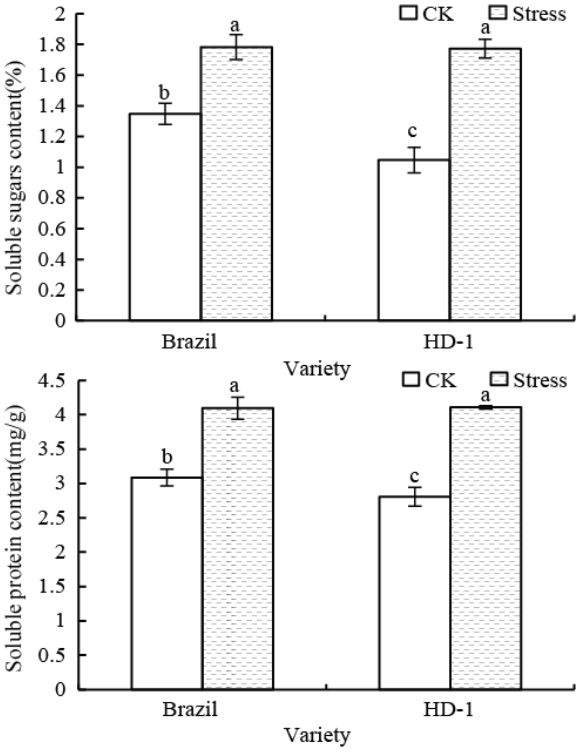


Fig. 21(b). Effect of salt stress on soluble sugars and soluble proteins content of Brazil and 'HD-1' banana leaves.

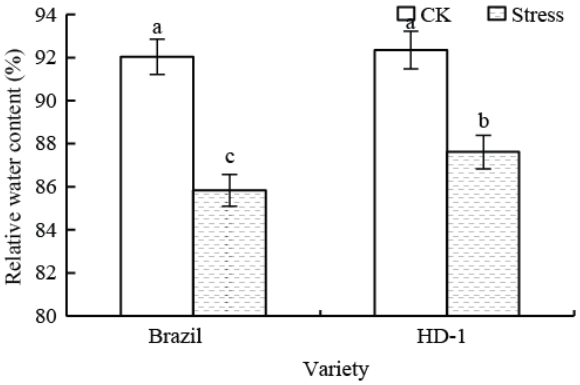


Fig. 22. Effect of salt stress on relative water content of Brazil and 'HD-1' banana leaves.

***Fusarium* wilt resistance comparison of 'HD-1' and Brazil Banana:** Yellowing phenomenon begins to appear on lower leaves of banana seedlings after inoculation about 15 days, spreads from leaf margin to the vein gradually, then the entire leaf wilts. 35-day duration of diseases extend upward from the bottom of the plant and the stem base near the surface cracks; bulbs become black or red-brown and roots become dark.

Table 12. are the resistant evaluation results of two banana varieties. The resistant evaluation result of 'HD-1' is 3, the resistance level being "S"; the resistant evaluation result of Brazil is 4, the resistance level being "HS". Therefore, banana strains 'HD-1' t shows stronger resistance to FusariumWilt than Brazil.

Discussion

Morphological classification of banana strain 'HD-1': The method of 15 integrated traits score proposed by the Simmonds system directly reflects the chromosome ploidy and sources of genome. It does not need large, expensive instruments and sophisticated experimental techniques, nor does it need big capital investment; this classification system can be put forward by simply being convenient, intuitive, operable and easily accepted by the people. Above all it is the main basis for the classification of banana. Due to the effects of environmental and cultivating conditions, genetic structure reflected by the phenotypic score and genetic does not share a strict correlation. Simmonds classification system, despite its rationality and status that it enjoys, has limitations on many aspects: such as a lack of a more detailed level of classification, germplasm identification, and registration of new varieties. Furthermore, his analysis of individual species is difficult to classify comprehensively and as such that he fails to reveal the banana evolutionary pathway, to name just a few. Therefore, we need further research to verify their genotype.

Karyotype analysis of banana strain 'HD-1': In the long process of evolution, the chromosome number and structure of the species change, which results in the reform of karyotypes, we can therefore conduct the evolutionary degree of species and genetic evolutionary relationship among species by karyotype analysis.

In the view of Stebbins, a famous botanical classification and evolutionary biologist, the basic trend of karyotype evolution of higher plants is the development from symmetry to asymmetry of direction; symmetric karyotype is often associated with more ancient or primitive plants whereas asymmetric karyotype frequently occurs in the more evolved or specialized plants. According to this particular school of thought, the banana strain 'HD-1' is a relatively primitive type in bananas evolution system. Reported "1B" Banana includes red bananas (AAA) and CRBP-39 (AAAB).

Gong Yulian analyzes the karyotype of red banana somatic cells and his result is "1B" type, the karyotype formula 3x=33=30 m+3 SM, in which 3 chromosomes are submetacentric, the rest metacentric chromosome and the fourth group are satellited chromosomes. Guo Jihua (2011) discovers that the karyotype formula of CRBP-39 banana is: 2n=4x=44=4L + 16M2 + 20M1 + 4S, all metacentric chromosome, chromosome number is 10. But the karyotype analysis results of this paper and the above two analysis differ greatly.

ISSR molecular markers of banana strain 'HD-1': Marker is an area that can be identified on the chromosome. Molecular markers are genetic markers based on the nucleotide sequential variation of genetic material between individuals, directly detecting biological differences on the DNA molecular level. Molecular markers have the following characteristics: (1) unaffected by environment, organization type, developmental stage; (2) high polymorphism and multiple tag number, throughout the entire genome; (3) there are many markers showing codominance to identify the homozygous and heterozygous genotype; (4) the DNA molecular marker technique is simple, fast, easy for long-term preservation material.

Resistance research of 'HD-1' banana seedling: Resistance is an important part of the evaluation of banana germplasm, and is closely related to banana production. In the actual production, low temperature, drought, salinity, plant diseases and insect pests and other stress factors are commonplace, banana varieties with strong resistance displays a stronger endurance, an increasing yield, and reduction in economic loss.

In this test, 'HD-1' and Brazil banana seedlings are treated with chilling stress, drought and salt stress respectively so as to obtain resistance strength of 'HD-1' by comparison with Brazil banana. Each treatment is selected for the main physiological indicators to measure, for which we have reached a preliminary conclusion. In subsequent species identification process, we must choose more physiological indicators of principal component analysis to calculate the resistance of membership functions to ensure that the results are accurate.

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