

# Influence Factors and Cell Structure Changes Related to Sugarcane Stem Tip Browning in Vitro Culture

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**Abstract** – Browning to death is a major limitation for sugarcane tissue culture. This study was mainly carried out to investigate the influence factors which relate to phenolic browning and analyze the relationship between cell structure and browning in sugarcane tissue culture through observing stem tip cellular compartments structure changes in the process of browning. The results showed that different axillary bud obtained obviously different induction survival rate, the first position bud got the highest survival rate, that is to mean the more fresh bud get the higher induction survival rate; the induction survival rate wasn't effected by different explants collecting season; pre-germination time of 4 weeks resulted higher induction survival rate, and obtained significant difference compared with other pre-germination time treatments. Different buds with different pre-germination time treatments had obvious different total phenolic content, the longer pre-germination time resulted the lower phenolic compounds accumulated in stem tip cells. The nucleus structure deformed, swollen mitochondria stretched, part of the vacuole membrane started to break down at the early browning stage; more serious plasmolysis appeared, a large number of lysosomes appeared in cytoplasm, organelles such as mitochondria completely decomposed, cell membrane, vacuole membrane, nuclear membrane and mitochondrial membrane broke down, cytoplasm completely dissolved, vacuoles mitochondria and other organelles completely decomposed at the later browning stage. This finding inferred that phenolic content, polyphenol oxidase activity as well as nucleus and mitochondria structural distortion and cell membrane broken are factors related to sugarcane stem tip browning in vitro culture. This work provides a reference for improving induction survival rate and lays a solid foundation for further study on mechanism of stem tip browning in vitro culture for sugarcane.

**Keywords** – Sugarcane, Stem Tip, Browning, Phenolic Content

## I. INTRODUCTION

Virus-free seedcane techniques have been progressed significantly in recent years and widely applied in

sugarcane growing areas all over the world [1]-[3]. Since this technology was introduced from Brazil in 1980s, shoot tip virus-free seedcane technique system including virus elimination [4], tissue culture and rapid propagation [5], pathogen detection [6], field propagation and cultivation [7], [8] had been established and successfully applied in most sugarcane growing areas in China.

Browning is considered to be a major limitation for plant tissue culture, which may cause an inhibition of growth, loss of regenerative capacity, and subsequent cell death [9]. Browning degree is affected by factors such as different plant genotype, growth conditions and explants obtained from which parts of the plant [10]. Non-enzymatic and enzymatic browning is considered as two main types of browning. Enzymatic browning was thought to be the major one in the process of plant tissue culture. The presence of phenolic compounds and oxidative enzymes is necessary during the process of enzymatic browning. In general, phenolic compounds and oxidative enzymes can be generated during normal plant metabolic processes, and the concentration will be increased in response to abiotic stresses. Moreover, the inevitably mechanical damage during explants preparation is thought to be a key factor attributed to browning during callus induction. Accumulation and oxidation of phenolic compounds thought to be the main reason for inhibition of growth and a decrease in the regeneration ability of plant cells [11]-[13]. Many technologies have been developed to control browning in plant tissue culture. Chemical additives such as antioxidants (ascorbic acid, melatonin, citric acid etc.) and sorbent (activated charcoal, polyvinylpyrrolidone, Polymeric Adsorbents, etc.) were put in the culture medium are commonly to prevent or reduce the deleterious effects of browning [10], [14], [15], and inclusion of phenylalanine ammonialyase inhibitors in the medium was also effective to overcome the harmful effects of browning by reducing polyphenols [12].

Lethal browning is also a serious obstacle in the maintenance of sugarcane tissue culture, which is considered to be a major cause of inducing survival rate decrease in shoot tip culture. The strategies used to overcome harmful effects of browning in sugarcane tissue culture including, addition of antioxidants and sorbent in the culture medium and transferring to fresh medium in a short intervals, etc. According to Huang et al.'s [16] report, soaking explants in sterile water prior to inoculating on MS medium with 2.0 mg/L 6-BA and 0.1 mg/L NAA

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was better to inhibit explants browning. Besides, addition of activated charcoal (0.02% is better, not higher than 0.05%) and polyvinylpyrrolidone in the culture medium is also effective to mitigate browning. Xian et al [17] reported that filter paper bridge culture in liquid medium minimized browning and got the best survival and induction rate contrast to other various culture methods. Liquid medium reduced the deleterious effects because of better diffusion of toxic substances around explants. In addition, transferring to fresh medium in a short interval was considered to be one of the effective strategies, although this method cost too much hand labor and medium materials.

Previous studies on sugarcane shoot tip tissue culture browning mainly focused on the harmful effects and controlling strategies. However, there is little report on factors and cellular structure changes related to browning in sugarcane shoot tip culture. This present study was mainly carried out to analyze the influences factors which relate to phenolic browning, and investigate stem tip cellular compartments structure alterations in the process of browning, in order to improve induction survival rate in sugarcane tissue culture and provide a reference for further study on mechanism of sugarcane stem tip browning in vitro.

## II. MATERIALS AND METHODS

### A. Plant Materials

The sugarcane variety ROC22 was selected as explants material in this study which was obtained from field of Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences. Single bud was collected in spring (March), summer (June), autumn (September) and winter (December) respectively. Four buds were taken as explants, the youngest buds but still has germination ability was marked as the 1st position bud, and 3rd, 5th, 7th position buds were marked in sequence from top to base direction. Then the single bud on 5 cm cane stem was pre-germinated at 38°C for 2 weeks, 3 weeks, 4 weeks and 5 weeks respectively. An orthogonal design of L16 (4<sup>4</sup>) was used to determinate the influences factors of browning with 3 factors and 4 levels (Table 1). One hundred buds were pre-germinated for each treatment.

Table 1. Experiment treatment factors and levels

Factors Levels	Explants collecting season	Pre- germination time	Different position buds
1	Spring (March)	2 weeks	1
2	Summer (June)	3 weeks	3
3	Fall (September)	4 weeks	5
4	Winter (December)	5 weeks	7

### B. Determination of Induction Survival Rate

Single bud was cultured on filter paper bridge in liquid medium after different pre-germination time. Culture medium was based on Murashige and Skoog (MS) medium (basal salts and vitamins) which was

supplemented with 30 g/L sucrose, 2.5m g/L 2,4-Dichlorophenoxyacetic acid and 0.1 mg/L naphthaleneacetic acid (NAA). Forty tips were cultured for each treatment. Induction survival rate was determined after 30 days.

### C. Polyphenol Oxidase (PPO) Activity Assay

Sugarcane single buds treated after different pre-germination time were prepared as aliquots for determination of PPO activity. The extraction was performed according to the method of Tang and Newton [18]. Shoot tip (0.2 g) was homogenized in 5ml of 0.1 M sodium phosphate buffer (pH 6.0) in ice bath. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C in a refrigerated centrifuge (BACKMANJ-20XPI). The supernatant was collected as crude extract and then the PPO activity was determined by the method of He et al. [19]. Half of 1 ml of crude extract was added to 4.5 ml of 0.05 M catechol (in sodium phosphate buffer). The absorbance of samples was measured at 410 nm for 3 min at 15 s intervals with a Genesys 20 visible spectrophotometer (Thermo Fisher Scientific, Somerset, NJ, USA). One unit of PPO activity was defined as the amount of enzyme that caused a change of 0.01 in the absorbance per minute [20].

### D. Determination of Total Phenolic Content

Sugarcane single bud with different pre-germination time was prepared for the determination of total phenolic content. The extraction was carried out according to the method of Gorinstein et al. [21]. Sugarcane stem tips (0.2 g) was homogenized and then added in 2 mL of 40 % methanol for extracting at 55°C for 30 min. Then the homogenate was centrifuged at 3000r/m for 10 min in a centrifuge (BACKMANJ-20XPI). The supernatant was collected as crude extract and then the total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu procedure as described by Eduardo et al. [22]. The total phenolic content was calculated based on a calibration standard curve of gallic acid and expressed as milligrams of gallic acid equivalents per 100 gram of fresh weight.

### E. Ultrastructural Analysis

Stem tip protomeristem was cultured on filter paper bridge in liquid medium. Scanning electron microscopy was performed every day for a month to investigate alterations in cellular structures during sugarcane callus culture. Samples was fixed for 1-2 h in 2.5% glutaraldehyde (Sigma-Aldrich Co.) and 4% formaldehyde (pH 7.2), and then post-fixed with 1% osmium tetroxide (OsO<sub>4</sub>) overnight at 4°C. The post-fixed tissues were washed three times in 0.1 M phosphate buffer (pH 7.2) and then dehydrated through a graded ethanol series (50, 70, 80, 90 and 100 % ethanol) for 15 min each. Samples were dried with a critical point dryer (CPD7501, VG. Microtech, East Sussex, UK), covered with gold by a SPIMODULE sputter coater (SPI Supplies Division of Structure Probe Inc., West Chester, PA, USA), and examined with a Quanta 450 scanning electron microscope (FEI, Brno, Czech Republic) [20].

### F. Statistical Analyses

Each experiment in the biochemical assays was designed according to a completely randomized design with 3 replicates per treatment. All data were analyzed by analysis of variance (SPSS18.0, Analytical software, IMB, USA) and means were compared based on the least significant difference test (LSD) at the 0.05 probability level.

## III. RESULTS

### A. Comprehensive Analyses of Influence Factors on Sugarcane Stem Tip Browning *in vitro* Culture

The influence factors analyses on phenol browning of sugarcane stem tip in the process of tissue culture *in vitro*

showed that different explants collecting season did not have significant effect on induction survival rate (Sig=0.135 > 0.05) and total phenolic content (Sig=0.162 > 0.05), but have significant effect on PPO activity (Sig =0.01 < 0.05). However, different position axillary bud and pre-germination time had significant effect on induction survival rate, PPO activity and total phenolic content (Table 2). The results of influence factors correlation analyses showed that total phenolic content had a significant negative correlation with induction survival rate ( $r=0.818$ ,  $p=0.01$ ). Besides, PPO activity also had a negative correlation with induction survival rate, but not significant (Table 3).

Table 2. Influence factors analyses on phenol browning of sugarcane stem tip *in vitro* culture

Resource	Dependent variable	Type III quadratic sum	Df	Mean square	F	Sig.
Calibration model	Induction survival rate	1910.360	9	212.262	24.533	.000
	PPO activity	10439.833	9	1159.981	16.529	.000
	Total phenolic compounds	766828.188	9	85203.132	9.139	.000
Intercept	Induction survival rate	58639.110	1	58639.110	6777.447	.000
	PPO activity	551265.333	1	551265.333	7855.040	.000
	Total phenolic compounds	9620356.688	1	9620356.688	1031.889	.000
Explants collecting season	Induction survival rate	51.152	3	17.051	1.971	.135
	PPO activity	1319.500	3	439.833	6.267	.001
	Total phenolic compounds	50555.229	3	16851.743	1.808	.162
Pre-germination time	Induction survival rate	378.329	3	126.110	14.576	.000
	PPO activity	7329.167	3	2443.056	34.811	.000
	Total phenolic compounds	439703.729	3	146567.910	15.721	.000
Different position buds	Induction survival rate	1480.879	3	493.626	57.053	.000
	PPO activity	1791.167	3	597.056	8.508	.000
	Total phenolic compounds	276569.229	3	92189.743	9.888	.000

Table 3. Influence factors correlation analyses on the survival rate of sugarcane stem tip *in vitro* culture

		Induction survival rate	Total phenolic compounds	Polyphenol oxidase activity
Induction survival rate	Pearson dependency	1	-.818	-.027
	Significant (both sides)		.001	0.857

### B. Effect of Different Factors on Induction Rate of Sugarcane Stem Tip *in vitro* Culture

The results showed that different axillary bud obtained obviously different induction survival rate, the first position bud got the highest survival rate, that is to mean the more fresh bud get the higher induction survival rate; the induction survival rate wasn't effected by different explants collecting season; pre-germination time 4 weeks resulted higher induction survival rate, and obtained significant difference compared with other pre-germination time treatment (Fig. 1).

### C. Effect of Different Factors on PPO Activity of Sugarcane Stem Tip *in vitro* Culture

PPO activity changed significantly in different axillary buds with different pre-germination time treatment, lower PPO activity were obtained in 1 - 6 position buds with 2 weeks pre-germinated treatment and in 3 - 6 position buds with 4 weeks pre-germinated treatment; while higher PPO activity were obtained in 1 - 3 position buds with 3 weeks

pre-germinated treatment and in 4 - 6 position buds with 5 weeks pre-germinated treatment (Fig. 2).

### D. Effect of Different Factors on Total Phenolic Content of Sugarcane Stem Tip *in vitro* Culture

There was no significant difference in total phenolic

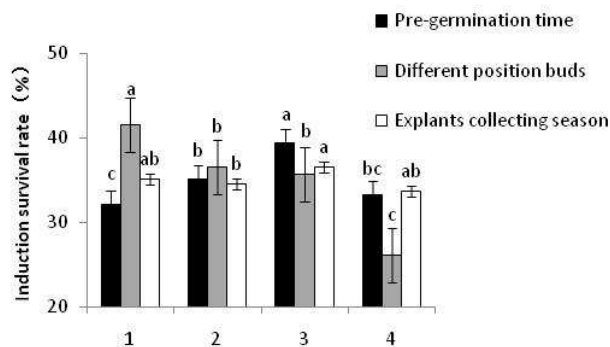


Fig. 1. Induction survival rate of stem apex in sugarcane *in vitro* culture affected by different influence factors

content among stem tip explants collected in different season. While different buds with different pre-germination time treatments had obvious different total phenolic content. Total phenolic content decreased with the increase of pre-germination time. Compared with the 2 weeks and 3 weeks pre-germination time treatments, pre-germination for 4 weeks resulted in low total phenolic content. Therefore, sugarcane axillary bud pre-germinated for 4 weeks is better for induction (Fig. 3).

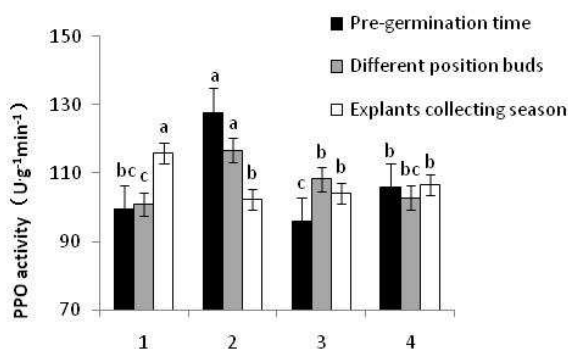


Fig. 2. Sugarcane stem tip polyphenol oxidase activity changes affected by different influence factors

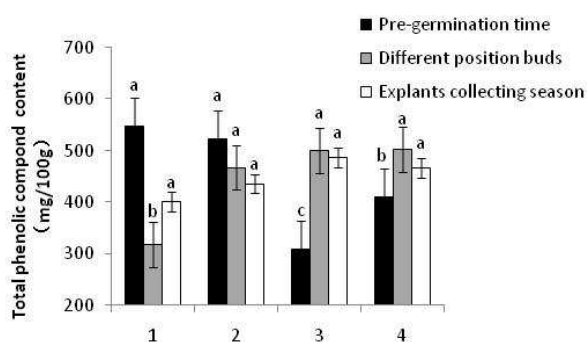


Fig. 3. Total phenolic compound content changes of sugarcane stem tip affected by different influence factors

#### E. Ultrastructural Analysis

Changes of subcellular characteristics of callus tissue were observed by electron microscope in the process of stem tip browning. On the first day, callus showed strong green color, cell wall was thin, tonoplast and plasmodesmata were clear, organelles, including a nucleus, mitochondria and chloroplasts in the cytoplasm were well-organized and very apparent (Fig.4). But slight plasmolysis appeared the next day. After four days, browning stem tip tissue cell's nuclei began to deform, partly swollen mitochondria appeared and the tonoplast began to degrade.

Browning callus after 14 days turned dark brown and exhibited numerous lysosomes were compared with non-oxidized callus. Nuclei deformation and plasmolysis were more obvious compared with that observed at four days. Some areas of the cell membrane system including tonoplast, nuclear envelope and mitochondrial membrane were broken, while the nuclear structure maintained normally in cells of non-oxidized callus.

After 20 days, the browning callus tissue was completely dark brown. Various callus cells were completely disorganized. Cell membrane, vacuole membrane, nuclear membrane and mitochondrial membrane broke down, cytoplasm completely dissolved, vacuoles mitochondria and other organelles completely decomposed.

#### IV. DISCUSSION

Browning during sugarcane tissue culture in vitro is affected by factors such as explants collecting season, different explants tissue, culture medium and intervals of subculture. The results of the present study showed that different explants collecting season resulted in different induction survival rate in sugarcane stem tip culture in vitro. Collecting explants in summer was better for callus induction, which is similar to the results of Lu's study [23]. Lu et al. [23] also reported that the best time for explants collection is from June to August when sugarcane is at elongation stage, while explants collected in winter was easy browning during in vitro culture and had a long induction period. We also found the stem tip which had relatively lower PPO activity and total phenolic content obtained a higher survival rate. Huang et al. [16] reported that higher phenolic compounds and enzyme content around stem tips resulted more serious browning in the process of tissue culture in vitro, compared with that using fresh leaves. But the influence factors of browning in single buds in vitro culture wasn't observed in that study.

Pre-germinated time also had a significant effect on induction survival rate of sugarcane tip in vitro culture. The previous study considered that pre-germination for 2 weeks would get a high induction survival rate, but the effect on induction survival rate of different pre-germinated times wasn't observed in their study [16], [23], [24]. Our study showed that pre-germination for 3-4 weeks obtained higher induction survival rate, which is different from that of the previous study.

Our results showed that PPO activity had no significant negative correlation with induction survival rate. It is probably because PPO activity was influenced by many factors such as light, PH and component of culture medium. Iqbal et al. [25] reported that PPO activity was high at PH 6.2, while low when PH is lower than 5.8 or higher than 7.0. PPO could also be activated at stress-inducing situations such as high temperature and strong light, which could result in rapid and serious browning of cultured tissue in vitro. For instance, sugarcane embryogenic cell turned brown in illumination 2500 lx, while green buds appeared in weaker light. Qin [24] reported that exogenous phytohormone caused browning by promoting the activity of PPO. Besides, the level of browning was affected by different concentration and combination of plant hormone. Phenolic compounds oxidized seriously in the treatment of BA combined with KT, while slightly in the treatment of BA or BA combined with NAA. Decreasing hormone concentration would decrease the level of browning.

Yang et al. [3] identified six kinds of polyphenols in the medium of sugarcane micropropagation in temporary immersion bioreactors, and found the polyphenols content had significant negative correlation with induction survival rate, which indicated that polyphenols contributed greatly to browning. The result is similar to that of in phalaenopsis tissue culture [26]. Yin et al. [26] reported that chlorogenic acid, catechol, caffeic acid and gallic acid, phydroxybenzoic acid, coumaric acid were correlated with browning, and the influence of benzoic acid on browning was neglectable. The total phenols were positively correlated with browning, which was also proved in Xu's report [27].

Sugarcane browning stem tips observed by electron microscope showed that the nucleus structure deformed, swollen mitochondria stretched, part of the vacuole membrane started to break down at the early browning stage; more serious plasmolysis appeared, a large number of lysosomes appeared in cytoplasm, organelles such as mitochondria completely decomposed, cell membrane, vacuole membrane, nuclear membrane and mitochondrial membrane broke down, cytoplasm completely dissolved, vacuoles mitochondria and other organelles completely decomposed at the later browning stage.

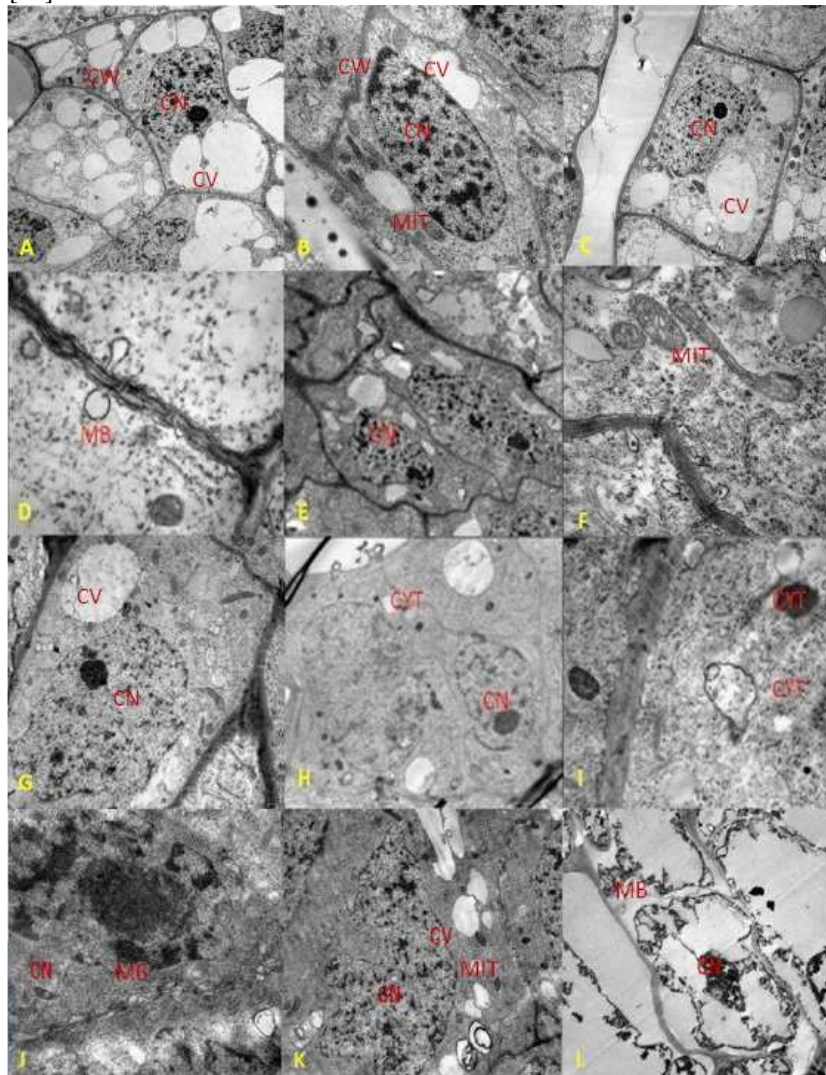


Fig. 4. Cell structure changes of sugarcane stem tip in the process of in vitro culture

A: Cell structure of sugarcane stem tip before inoculation; B: Cell structure of sugarcane stem tip 1 day after inoculation; C, D: Cell structure of sugarcane stem tip 2 days after inoculation; E, F: Cell structure of sugarcane stem tip 4 days after inoculation; G: Cell structure of normal sugarcane stem tip after 10 days of in vitro culture; H, I: Cell structure of browning sugarcane stem tip after 10 days of in vitro culture; J: Cell structure of normal sugarcane stem tip after 14 days of in vitro culture; K: Cell structure of browning sugarcane stem tip after 14 days of in vitro culture; L: Cell structure of browning sugarcane stem tip after 20 days of in vitro culture. Note: CW: cell wall; CN: nucleus; CV: vacuole; MIT: mitochondria; MB: lysosome; CYT: dictyosome.

## V. CONCLUSION

This finding inferred that phenolic content, polyphenol oxidase activity as well as nucleus and mitochondria

structural distortion and cell membrane broken are factors related to sugarcane stem tip browning in vitro culture. This work provides a reference for improving induction survival rate and lays a solid foundation for further study on mechanism of stem tip browning in vitro culture for sugarcane.

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