

Full Length Research

# Tannin dynamics in hypocotyls and pericarps of *Aegiceras corniculatum* fruits during dry storage

Ping Xiang<sup>1</sup>, Yi-Ming Lin<sup>1\*</sup>, Shu Ju<sup>2</sup>, Cheng Xiang<sup>1</sup> and Peng Lin<sup>1</sup>

<sup>1</sup>Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, School of Life Sciences, Xiamen University, Xiamen 361005, China.

<sup>2</sup>Department of Biology, University of Miami, Coral Gables, FL 33124, USA.

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In this study, we investigated the changes in total phenolics, extractable condensed tannins (ECT), protein noncovalent bound condensed tannins (PNBCT) and irreversible covalent bound condensed tannins (ICBCT) in the hypocotyls and pericarps of *Aegiceras corniculatum* fruits during dry storage. The loss of moisture content and survival rate, as indicators of the viability and quality of plant tissues, were also monitored during the dry storage. Great amount of free condensed tannins combined with proteins in the senescent pericarps during the process of deteriorative reactions associated with dry storage, which contributed to the rapid increase of PNBCT characterized by the sigmoid model. However, there was a rapid loss of total phenolics and ECT from pericarps during the first 9 days of the dry storage, with 37.3% loss of ECT due to the transformation of PNBCT. PNBCT of the hypocotyls also fitted a sigmoid model with dry storage time, and was related to the viability of the hypocotyls. During the dry storage, most of the disappeared ECT of hypocotyls formed complexes with protein, and the total condensed tannins (TCT) did not decline during the 27 days dry storage. The distribution of the oligomers and polymers in the condensed tannins was significantly different between the fresh and dried hypocotyls of *A. corniculatum*. The oligomers with lower degree of polymerization (DP) appeared to have higher capacity of binding to proteins than the polymers with high DP in plant tissues during the dry storage. The changes of hydroxy pattern of tetramers in the fresh and dried hypocotyls showed that the proteins would be selective binding those flavan-3-ol oligomers with more hydroxyl in plant tissues with deteriorative reactions during dry storage. However, the hydroxy pattern of monomers was not significantly different between the fresh and dried hypocotyls. Our results indicated that few flavan-3-ol polymers reacted with proteins in hypocotyls of *A. corniculatum* associated with the deteriorative reactions during the dry storage.

**Key words:** Tannin, polymerization degree, hydroxy pattern, dry storage, fruit, Mangrove, *Aegiceras corniculatum*.

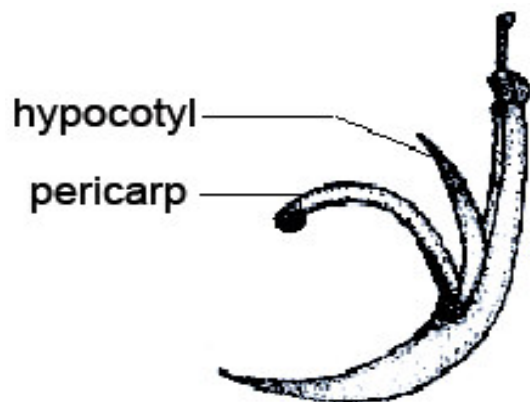
## INTRODUCTION

Tannins known as the group of phenolic compounds are significant plant secondary metabolites. They occur in plant leaves, roots, wood, bark, fruits and buds (Kraus et al., 2003), which were estimated to be the fourth most abundant biochemical substances produced by vascular plant tissue after cellulose, hemicellulose and lignin (Hernes and Hedges, 2000). Tannins in vascular plants

occur in two types, condensed tannins and hydrolyzable tannins. Hydrolyzable tannins consist of simple phenolic acids such as gallic acid esterified to a core polyol, typical glucose. Condensed tannins are polymers of flavonoid units (Monagas et al., 2010). Some plants evolved tannins production as a defense strategy, against invasion by pathogenic bacteria and fungi and against being eaten by insects and herbivores, with the mechanism being the protein precipitating properties of tannins (Bi et al., 1997).

Tannins have long been known to react with and bind to proteins under both non-oxidizing and oxidizing conditions. Under nonoxidizing conditions, tannins form noncovalent complexes with proteins through hydrogen

\*Corresponding author. E-mail: [linym@xmu.edu.cn](mailto:linym@xmu.edu.cn), [linym1967@yahoo.com.cn](mailto:linym1967@yahoo.com.cn). Tel: 86-592-2187657. Fax: 86-592-2181015.



**Figure 1.** The longitudinal section of a mature *A. corniculatum* fruit.

bonding or hydrophobic interactions. The very nonpolar hydrolyzable tannin (pentagalloylglucose) precipitates by forming a hydrophobic boat around the protein, whereas the much more polar condensed tannins (EC<sub>16</sub>-C) forms hydrogen-bonded cross-links between protein molecules (Hagerman et al., 1998). The noncovalent nature of the complexes is demonstrated by release of both protein and polyphenolics when the precipitate is treated with protein denaturants such as sodium dodecyl sulfate (SDS) (Hagerman and Butler, 1980). Under oxidizing conditions, tannin reacts with protein to form tannin-protein complexes through covalent bond. However, these complexes are resistant to disruption by protein denaturants (Stern et al., 1996; Chen and Hagerman, 2004). At the cellular level, tannins have been reported in cell walls, intercellular space, and vacuoles (Kraus et al., 2003). Tannins are unstable phenolic compounds and change rapidly in plant tissues after excision. Protein-tannin complexes may form in living plant tissues prior to leaf fall (Gallet et al., 1999). During plant senescence, tannins may mix with cell contents to form additional recalcitrant complexes. Also, in harvested plant samples, condensed tannins (proanthocyanidins) may bind to plant material, with condensed tannins binding more strongly to protein than fibre (Kandil et al., 2004; Lin et al., 2010), which is significantly affected by plant sample drying conditions (Makkar and Singh, 1995; Palmer et al., 2000), plant sample preservation (Dalzell et al., 1997) and sample grinding (Guzman-Maldonado et al., 1996). Binding to protein is the most important change for tannins in plant tissues. When the plant samples were either oven-dried or frozen-dried, the non-extractable condensed tannins bound mostly to plant proteins and almost all of the condensed tannins bound to protein in oven-drying leaves of *Gliricidia sepium* (Jackson and Barry, 1996; Mupangwa et al., 2000).

There are several mechanisms by which condensed tannins can react with macromolecules in the plant tissues including forming noncovalent complexes with proteins and initiating oxidative processes mediated by

quinines, leading to irreversible covalent links with protein and other macromolecules (Haslam, 1986; Makkar and Singh, 1995; Hagerman et al., 1998; Bourvellec et al., 2004). There have been studies on the extractable and bound condensed tannin concentrations and the effects of sample drying, storage, or extraction on the yield of plant condensed tannins, mainly in plant leaves (Terrill et al., 1992; Makkar and Singh, 1995; Jackson and Barry, 1996; Dalzell and Shelton, 1997; Makkar et al., 1999; Mupangwa et al., 2000; Palmer et al., 2000; Zhang et al., 2010). However, there is scant information about the binding processes of free condensed tannins in plant tissues during dry storage and with deteriorative reactions. The objective of this research was thus to study possible changes in total phenolics, extractable condensed tannins (ECT), protein noncovalent bound condensed tannins (PNBCT) and irreversible covalent bound condensed tannins (ICBCT) in the hypocotyls and pericarps (Figure 1) in *Aegiceras corniculatum* fruits. *A. corniculatum* is a major mangrove species in the intertidal zone of subtropical coast, China. The binding processes of free condensed tannins in plant tissues were also discussed.

## MATERIALS AND METHODS

### Fruits treatments

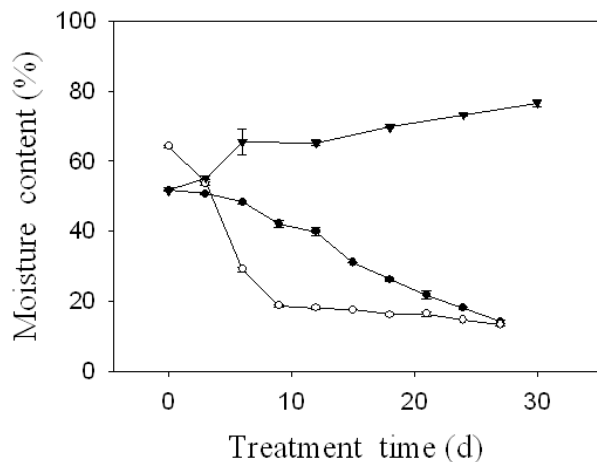
The mature fruits of *Aegiceras corniculatum* were collected in August 2003 from the mangrove forest at Jiulong River Estuary (24°24'N, 117°55'E), Fujian, China. The mean weight of the collected fruits was  $0.893 \pm 0.029$  g. The fruits were taken to laboratory immediately and dry storage at room temperature in a dry room, avoiding sunlight. Total of 50 fruits were randomly taken out every 3 days, with 20 fruits used for tannin measurement and 30 for pot culture to determine their germination rates. The salinity for the culture medium was  $8.0 \pm 0.5\%$ , which was similar to the natural environment at Jiulong River Estuary. These comparing samples were randomly selected and prepared for analysis after 0, 3, 6, 12, 18, 24 and 30 days. All the treatment was three replicates respectively. A sub-sample of the hypocotyls and pericarps was oven-dried at 105° for calculating the dry weight of samples and the moisture contents.

### Chemicals

All chemicals were of Analytical Reagent (AR) purity grade. (+)-catechin and tannic acid standard was obtained from Sigma. Sephadex LH-20 was purchased from Amersham (USA).

### Extraction

For the dry storage and pot culture experiments, duplicate 100 mg hypocotyls and pericarps of fruits were weighed and ground in 70% (V) acetone solution at iced temperature. The samples were then transferred into 10 ml screw-top polyallomer centrifuge tubes and extracted three times with 5 ml 70% (V) acetone solution. The supernatants were combined to a 50 ml conical flask. After removing a 5 ml of the pooled extracts for total phenolics analysis, the remainder of the extract was added with 0.001 M ascorbic acid



**Figure 2.** The changes of moisture content in hypocotyls of culture experiment (▼), hypocotyls (●) and pericarps (○) of fruits during dry storage.

to minimize oxidation (Schofield et al., 1998). The acetone was evaporated by  $N_2$  stream and the water fraction was purified by liquid/liquid extraction with EtOAc 3 times to remove chlorophyll and monomer phenols. The aqueous fractions were further analyzed by vanillin/HCl assay and butanol/HCl assay. To extract protein noncovalent bound CT as described by Terrill et al. (1992), 15 ml SDS solution (10 g litre<sup>-1</sup> SDS and 50 g litre<sup>-1</sup> 2-mercaptoethanol in 10 mM Tris/chloride, pH 8.0) was added to the solid residue from the first extraction. The tubes were shaken on a vortex mixer and in boiling water for 45 min. Tubes were cooled to room temperature and centrifuged at 5000 × g for 15 min, and the supernatant was poured into another 50 ml conical flask. This process was repeated 3 times until the supernatants were combined and increased to 50 ml total volume. ICBCT was determined directly on the residue remaining from the extraction of PNBCT, as described below.

### Analysis process

Total phenolics in the crude extracts were measured with the Prussian blue method (Graham, 1992), using tannic acid as the standard. The ICBCT measured procedure and the butanol/HCl for ECT were described by Terrill et al. (1992), with purified *A. corniculatum* leaf condensed tannins as the standard. The vanillin/HCl method (Sun et al., 1998) was applied for sample extract solutions with catechin as the standard.

### Extraction of free CT and PNBCT with 70% acetone solution *in vitro*

To determine the extraction of free CT and PNBCT with 70% acetone solvent *in vitro*, we used bovine serum albumin (BSA) as a model protein (Hagerman et al., 1998; Chen and Hagerman, 2004). Lyophilized condensed tannins (3.0 mg) purified from *A. corniculatum* leaves and BSA was 1:1 (w/w) mixed and ground into fine powder. One part of the powder was extracted three times with 5 ml 7:3 (v/v) acetone / water solution. The supernates were combined to a 50 ml conical flask. Another part was dissolved in 1 ml metal-free distilled water and slight protein-CT precipitation occurred rapidly. The solutions were mixed and allowed to stand at room temperature for about 1 h. and then centrifuged at 5000 × g for 15 min. The supernatant was discarded and the surface of the

pellet and the walls of the tube were washed 3 times with metal-free distilled water. The precipitate was extracted three times with 5 ml 7:3 (v/v) acetone/water solution too. The supernates were combined to a 50 ml conical flask. The residue remaining from the extraction was dissolved in 5 ml SDS solution, boiling water bathed for 45 min. The acetone was evaporated by  $N_2$  stream and the aqueous fractions were further analyzed by butanol/HCl assay.

### MALDI-TOF MS sample preparation

The spectra were recorded on a Bruker Reflex β. The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm, and the duration of the laser pulse was 3 ns. In the positive reflectron mode, an accelerating voltage of 20.0 kV and a reflectron voltage of 23.0 kV were used. The spectra of condensed tannins were obtained from a sum of 100 - 150 shots and were calibrated using Angiotensinα (1046.5 MW), Bombesin (1619.8 MW), ACTHclip18 - 39 (2465.2 MW), and Somatostatin28 (3147.47 MW) as external standards.

2, 5-dihydroxy benzoic acid (DHB, 10 mg/ml 30% acetone solution) was used as a matrix based on the results of Pasch and Pizzi (2002). The four condensed tannin samples (10 mg/ml 30% acetone solution) were deionized by strong cation-exchange resin (Dowe × 50 × 8 - 400, about 50 mg in 1 ml analyte solution) and then mixed with the NaCl (0.52 mg/ml aqueous solution), or CsCl (1.52 mg/ml aqueous solution) solution at a volumetric ratio of 1:1. The analyte/ cationization reagent solution were mixed with matrix solution at a volumetric ratio of 1:3 after the matrix solution was deionized by strong acidic cation-exchange resin respectively. The analyte/matrix solution applied (1 μl) to steel target, dried at room temperature. The Amberlite IRP-64 cation-exchange resin and Dowe × 50 × 8 - 400 cation-exchange resin, equilibrated in 70% acetone solution at room temperature, was used to deionize the analyte and matrix solution. The cationization reagent solution containing almost equally cations (0.045 mol/l) was mixed with the analyte solution to promote the formation of a single type of ion adduct ( $[M + Na]^+$ , or  $[M + Cs]^+$ ).

### NMR analysis

<sup>13</sup>C NMR spectra were recorded in CD<sub>3</sub>COCD<sub>3</sub>-D<sub>2</sub>O mixture with a Unity 500 spectrometer at 150 MHz (proton decoupling mode for carbon) (Behrens et al., 2003).

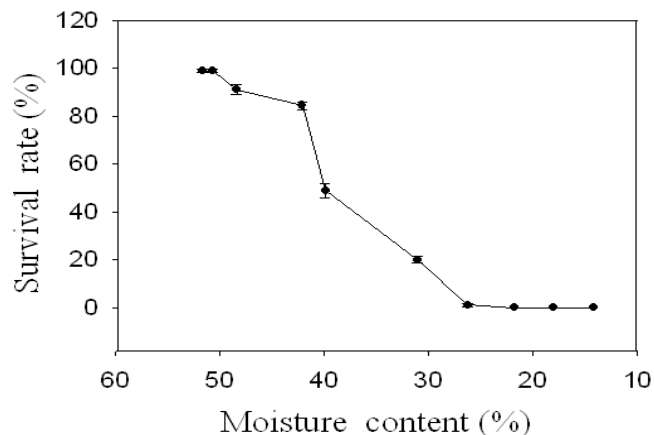
### Statistical analyses

All measurements of the total phenolics, ECT, PNBCT, and ICBCT were replicated 3 times and all data were analyzed using one-way analysis of variance (ANOVA) (SPSS12.0 for Windows).

## RESULTS

### Moisture content and survival of the fruits

The dynamics of the moisture content of hypocotyls and pericarps during dry storage was significantly different (Figure 2). The decline in moisture content closely followed exponential decay kinetics in pericarps  $\{r = 0.98$ , in  $[\text{moisture content}] = 10.10 + 54.13 \exp(-0.176(t))$ , where  $t = \text{treatment time (d)}\}$ . The significant decrease of moisture content occurred in the initial 9 days of storage



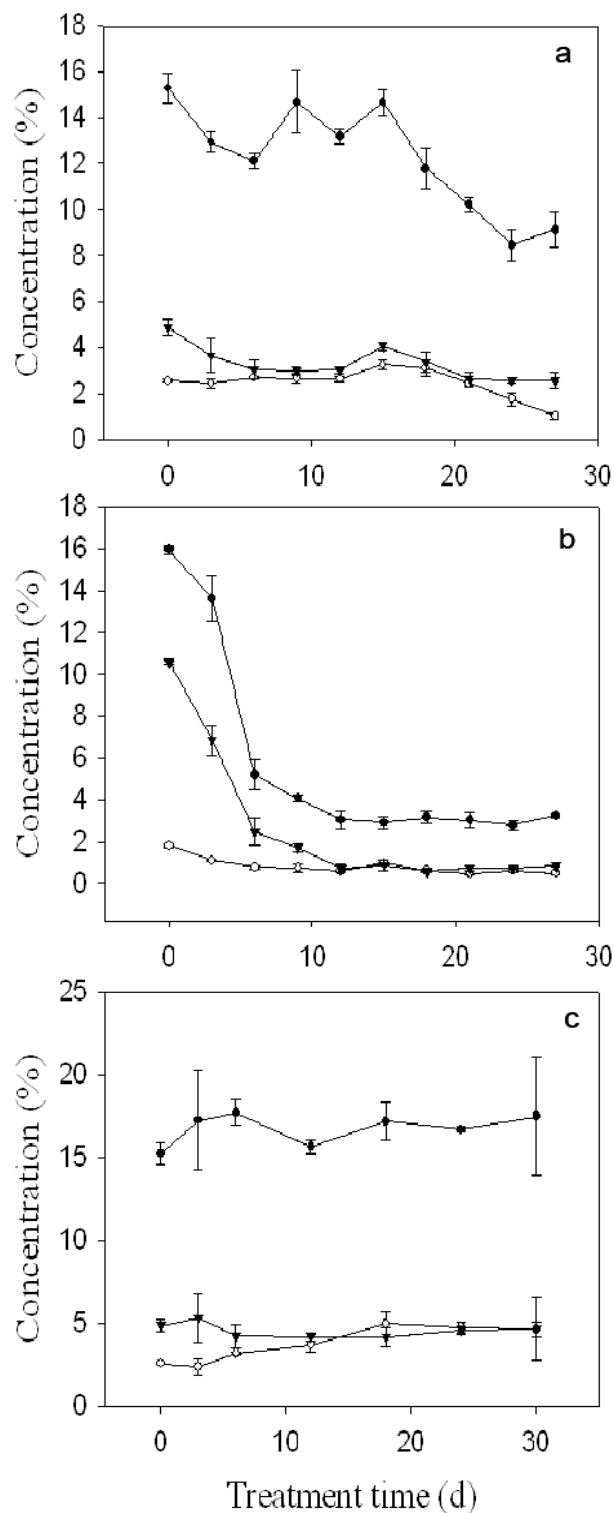
**Figure 3.** The decrease of survival rate (% germination) of the fruits with moisture content decline in hypocotyls during dry storage.

from 64.2% in fresh pericarps to 18.8%, and then moisture content declined smoothly. The change of moisture content in hypocotyls during dry storage followed linear kinetics  $\{r = 0.99, \text{in } [\text{moisture content}] = 54.94 - 1.523(t)\}$ , decreasing from 51.6 to 14.2%, and approached the level of pericarps at last ( $t = 1.02, p < 0.37$ ). However the moisture content of the hypocotyls increased from 51.6% to 76.5% during 30 days culture treatment, following closely linear kinetic  $\{r = 0.94, \text{in } [\text{moisture content}] = 55.14 + 0.764(t)\}$  (Figure 2).

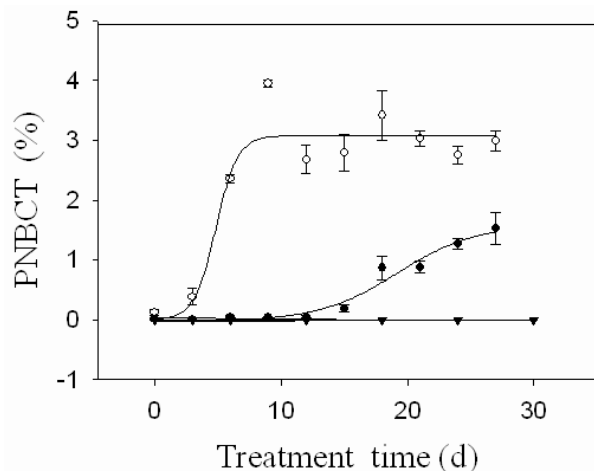
The survival rates of *A. corniculatum* fruits declined correspondingly with the loss of moisture in the hypocotyls during dry storage (Figure 3). When the moisture content of hypocotyls reduced from 51.6 to 42.1% during the first 9 days, the survival rates of fruits decreased slowly from 99.0 to 84.4%. However, when the moisture content further decreased to 26.3%, which is about a half of the initial moisture content in the fresh fruits, the survival rate decreased dramatically to 0 at 18 days.

### Total phenolics and condensed tannin content

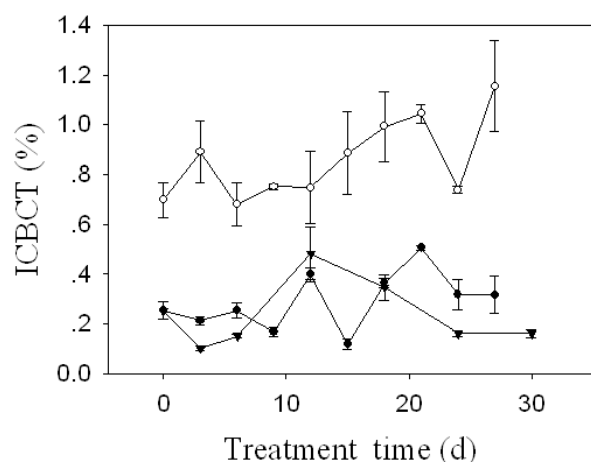
The content of total phenolics and condensed tannins in the hypocotyls and pericarps during dry storage showed a decline trend (Figure 4), in contrast with the trend observed for culture hypocotyls. Total phenolics in the pericarps decreased dramatically from 15.95 to 3.05% during the first 12 days storage and then remained relatively stable. This overall decrease curve closely followed exponential decay kinetics  $\{r = 0.96, \text{in } [\text{phenolics}] = 2.47 + 14.47 \exp(-0.197(t))\}$ . The hypocotyls showed a different decline trend for total phenolics from the pericarps. The rapid decline of total phenolics in the hypocotyls occurred during the last 12 days, from 14.67% at the 15<sup>th</sup> day to 9.13% at the 27<sup>th</sup> day. For the culture



**Figure 4.** The changes of extractable total phenolics (●) and extractable condensed tannins (ECT) [analyzed by butanol/HCl (▼) and by vanillin/HCl (○)] in hypocotyls (A), pericarps (B) during dry storage and culture hypocotyls (C). Extractable total phenolics were determined with the Prussian blue method using tannic acid as the standard. Condensed tannins were determined with butanol/HCl method using *A. corniculatum* leaf CT as the standard and vanillin/HCl using (+)-catechin as the standard.



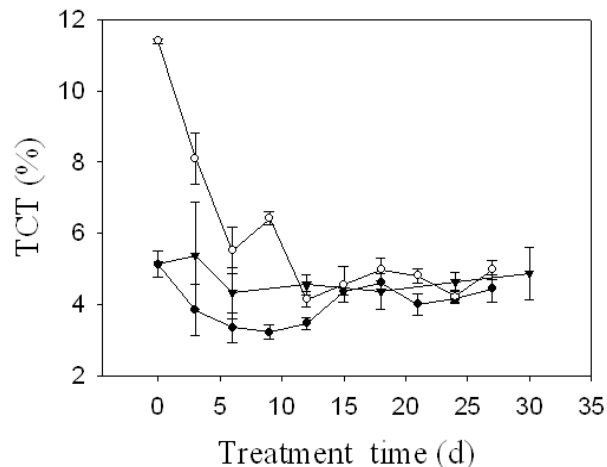
**Figure 5.** The protein noncovalent bound condensed tannins (PNBCT) in hypocotyls (●), pericarps (○) during dry storage and culture hypocotyls (▼).



**Figure 6.** The irreversible covalent bound condensed tannins (ICBCT) in hypocotyls (●), pericarps (○) during dry storage, and culture hypocotyls (▼).

hypocotyls, total phenolics changed insignificantly during the entire 30-day experiment period.

The changes of butanol/HCl CT (measured by butanol/HCl assay, Figure 4) were similar to total phenolics in all three samples. During the dry storage, the change of butanol/HCl CT in pericarps closely followed exponential decay kinetics  $\{r = 0.99, \text{ in } [\text{butanol/HCl CT}] = 0.50 + 10.39 \exp(-0.225(t))\}$ . The extractable butanol/HCl CT in hypocotyls decreased rapidly from 4.06 to 2.60% during the last 12 days. For the cultured hypocotyls, the butanol/HCl CT didn't change during the entire experiment period. There were similar trends for Vanillin/HCl CT (Figure 4) between the hypocotyls and the pericarps during the dry storage. For the pericarps, the content of Vanillin/HCl CT decreased dramatically from 1.82% in the initial fresh pericarps to 0.73% at the 9<sup>th</sup> day. A sharp



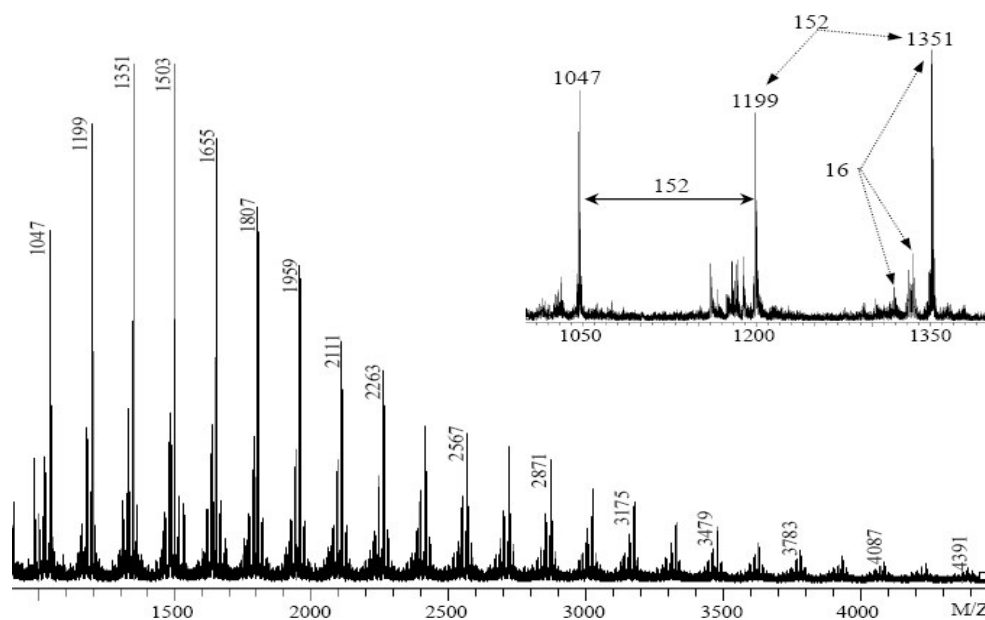
**Figure 7.** The changes of total condensed tannins (TCT) in hypocotyls (●), pericarps (○) during dry storage and culture hypocotyls (▼).

decrease in Vanillin/HCl CT extracted from hypocotyls occurred during the 12 day dry process. However, for the cultured hypocotyls, the content of Vanillin/HCl CT increased from 2.58 to 4.65%. The protein noncovalent bound CT (PNBCT) of pericarps with the storage time closely fit the sigmoid dynamics  $\{r = 0.95, \text{ in } [\text{PNBCT}] = 3.05 / (1 + \exp(-(t - 4.85)/0.852))\}$ . They increased sharply in rate of 33.18  $\times$ , from 0.12% in initial fresh pericarps to 3.95% at the 9<sup>th</sup> day and remained at the high level at least until the 27<sup>th</sup> day (Fig. 5). The PNBCT of hypocotyls also fitted a sigmoid dynamics  $\{r = 0.98, \text{ in } [\text{PNBCT}] = 1.57 / (1 + \exp(-(t-19.20)/2.937))\}$ . However the concentration remained at a negligible level during the first 12 days, and then increased rapidly to 1.59% at the end of storage. However, no PNBCT were detected in the cultured hypocotyls over the 30 day culture treatment (Figure 5).

The irreversible covalent bound CT (ICBCT) of pericarps increased from 0.70 to 1.15% over the 27 days of dry storage ( $t = 0$  sample vs.  $t = 27$  days sample,  $t = -2.335$ ,  $P < 0.05$ ), whereas, the ICBCT of hypocotyls remained at lower level than pericarps and unchanged during dry storage (Figure 6). TCT content of the pericarps decreased exponentially from 11.41 to 4.98% during storage (Figure 7), and followed an exponential decay model  $\{r = 0.97, \text{ in } [\text{TCT}] = 4.61 + 6.84 \exp(-0.248(t))\}$ . However, TCT of hypocotyls remained the constant during storage. TCT in culture hypocotyls had the same result.

#### MALDI-TOF mass spectrum of condensed tannins from fresh mature hypocotyls

Extractable condensed tannins (ECT) extracted from fresh mature hypocotyls of *A. corniculatum* gave a high quality MALDI-TOF spectrum (Figure 8) in the case of



**Figure 8.** MALDI-TOF mass spectrum  $[M + Cs]^+$  of condensed tannins extracted from fresh mature hypocotyls and the enlarged spectrum of  $m/z$  1000~1400.

$Cs^+$  as a cationization reagent for MALDI. ECT gave clear spectra showing the oligomers and polymers of building units from trimer to tetradecamer ( $m/z$  4391.4), and oligomer or polymer series with masses of the repeat units of 152.0 Da. For each multiplet, a substructure with mass decrements of 16 Da appeared. These masses could be explained by heteropolymers of repeating flavan-3-ol units being lack of an additional hydroxyl group ( $\geq 16$  Da) at the 5' position of the B-ring as C/EC.

According to the mass spectrum, we concluded that the ECT from fresh mature hypocotyls of *A. corniculatum* mainly contains GC/EGC units. In addition to the dominant flavan-3-ol units, a small quality of C/EC units and galloyl flavan-3-ol units occur in this condensed tannins. The MALDI-TOF mass spectra (Figure 8) also showed a series of oligomers containing one or two A-type linkage, which are present in low concentrations. Unfortunately, another series of flavan-3-ol oligomers and polymers with mass increments of 16 Da could not be explained.

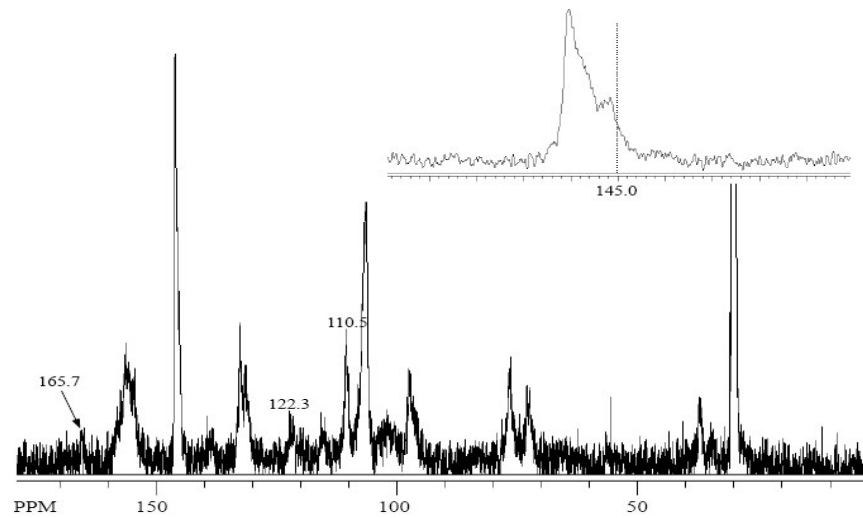
The ECT from fresh mature hypocotyls of *A. corniculatum* was also characterized by  $^{13}C$ -NMR in 95% deuterated acetone solution (Figure 9). The results of chemical structure properties by  $^{13}C$ -NMR was consistent with those by MALDI-TOF MS. C/EC units in condensed tannins produce peaks at 115 - 116 ppm for C-2' and C-5' and at 119 ppm for C-6', while PD units have no intensity in this region. No obvious peaks being observed in the spectrum of the ECT from fresh mature hypocotyls of *A. corniculatum* showed that GC/EGC was the dominant flavan-3-ol units in this condensed tannins. The C-2 region occurs at 75-85 ppm in the  $^{13}C$ -NMR of

condensed tannins, with 80 ppm being the dividing line between *cis* and *trans* structures. C-2 (*trans*) region at 80-85 ppm is relative weak in this case, reflecting the low proportion of *trans* structures. These results indicated that epigallotannins is the main flavan-3-ol units in the ECT from fresh mature hypocotyls of *A. corniculatum*. The presence of the galloyl group at C-3 could be obtained from the peaks at 165.7, 122.3 and 110.5 ppm.

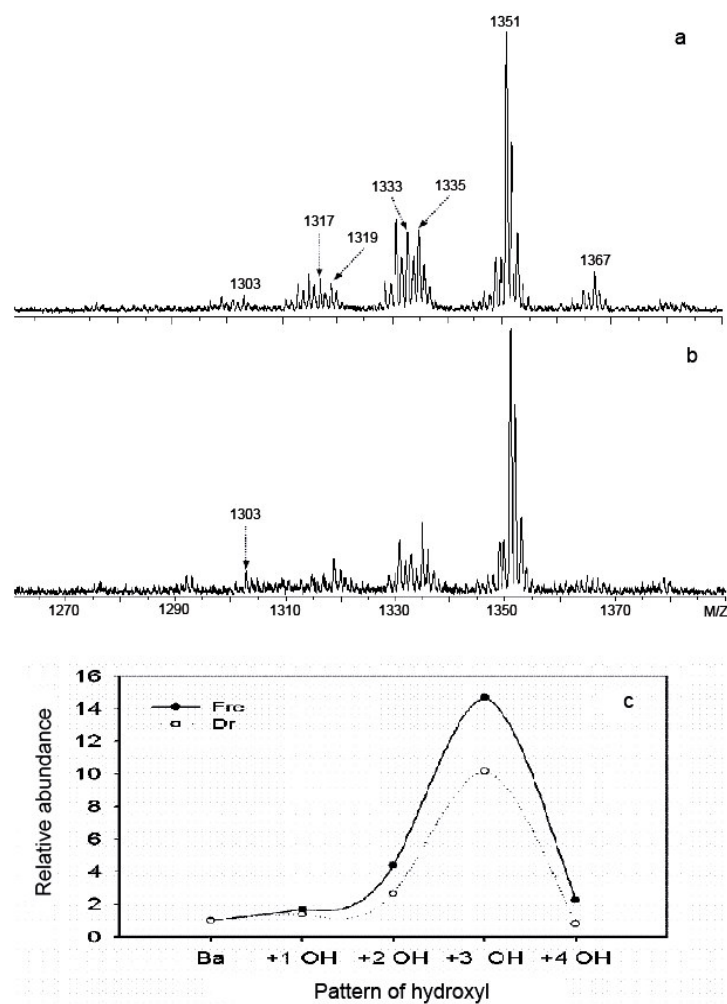
### MALDI-TOF mass spectrum of condensed tannins from dried hypocotyls

The chemical structure properties of ECT from dried hypocotyls including, distribution of oligomers and polymers, and the average degree of polymerization (DP) was different from the ECT from the fresh hypocotyls. The details about the pattern of hydroxyl could be observed from the tetramers (Figure 10) and nonamers (Figure 11) in the ECT from fresh and dried hypocotyls. It could be seen from the change of the hydroxy pattern of tetramers in the fresh mature hypocotyls and dried hypocotyls that the proportion of the oligomers with more hydroxyl decreased in tetramers during dry storage. However, this change of the hydroxy pattern of nonamers in the fresh mature hypocotyls and dried hypocotyls could not be obviously observed.

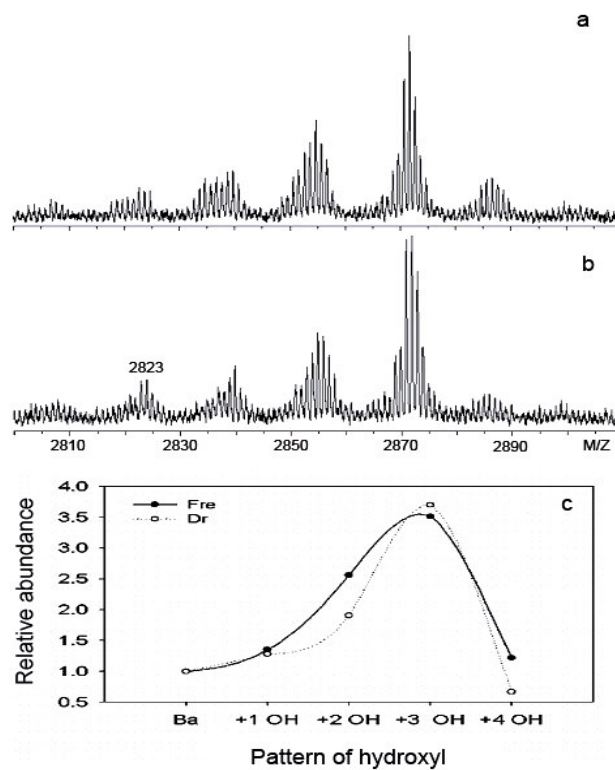
Figure 12 showed the change of the distribution and average DP of the oligomers and polymers in the fresh mature hypocotyls and dried hypocotyls. In contrast with the increment of the composing proportion of the



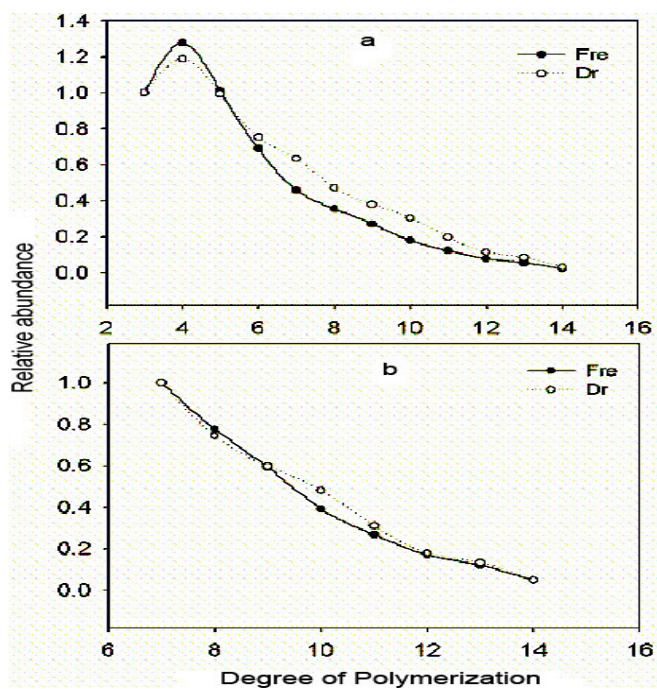
**Figure 9.** The  $^{13}\text{C}$ -NMR spectrum of condensed tannins extracted from fresh mature hypocotyls and the enlarged spectrum of 140 -150 ppm.



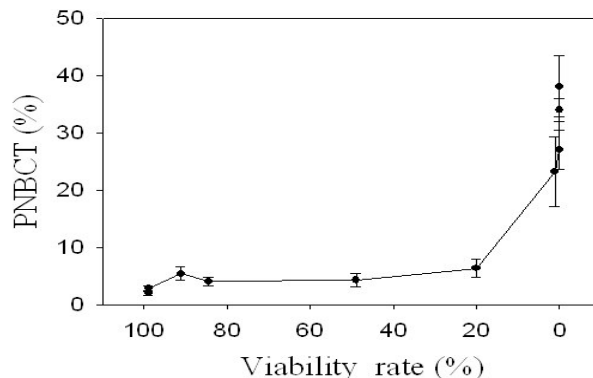
**Figure 10.** The change of the hydroxy pattern of tetramers in the fresh mature hypocotyls (Fre, A) and dried hypocotyls (Dr, B), the relative abundance based on the peak at m/z 1303 as 1 unit in the curve (C).



**Figure 11.** The change of the hydroxy pattern of nonamers in the fresh mature hypocotyls (Fre, A) and dried hypocotyls (Dr, B), the relative abundance based on the peak at  $m/z$  2823 as 1 unit in the curve (C).



**Figure 12.** The change of the distribution of the oligomers and polymers in the fresh mature hypocotyls (Fre) and dried hypocotyls (Dr), the relative abundance based on the trimers (A) or heptamers (B) as 1 unit in the curve.



**Figure 13.** The cumulating of PNBCT in hypocotyls during viability losing for desiccation.

polymers in ECT from the dried hypocotyls, the proportion of the oligomers with relative lower DP in ECT from the dried hypocotyls was lower than those in ECT from the fresh hypocotyls. This change of the distribution of oligomers and polymers would lead to increment of the average DP of the ECT from the dried hypocotyls. Figure 12 (B) further indicated that relative proportion of the polymers from heptamers to tetradecamers in ECT from the dried hypocotyls was not significant different from the ECT from the fresh hypocotyls. These results showed that the oligomers with relative lower DP in ECT were lost more than longer chain ECT in the hypocotyls during dry storage.

## DISCUSSION

*A. corniculatum* is a major mangrove species in the intertidal zone of subtropical coast in China (Lin et al., 2007). Since *A. corniculatum* is cryptoviviparous, the embryo germinates within the fruit and the hypocotyle comes out of the seed coat but it does not pierce the pericarp. Instead, the pericarp splits during seedling establishment. For the mature fruits, the pericarps were yellow and senescent obviously. The significant difference in the moisture content between the pericarps and hypocotyls during dry storage indicated that, besides protecting hypocotyls from light, pericarps prevented air flowing in hypocotyls along with the compact hypocotyls epidermis.

Desiccation was one of the most critical factors responsible for fruit mortality. The survival rate of fruits is like the recalcitrant seeds that they suffered desiccation mortality when they were allowed to dry below some critical moisture level (Figure 3). With the processes of moisture content decrease and viability losing, the tissues and cells of fruits suffered deteriorative reactions and this allow mixing of vacuolar and cytoplasmic contents (Haslam, 1986; Gallet et al., 1999), which resulted in binding of total phenolics, ECT and influence greatly on

the amount of PNBCT and ICBCT. In the 70% acetone extraction mixtures, very little CT co - precipitated with the protein (BSA) because the protein hardly dissolved in this solvent, and 97.70% of the added 3.0 mg CT was recovered. This solvent did not dissolve the CT-protein precipitate that formed in metal-free distilled water through noncovalent bond as well. The results suggest that the 70% acetone solvent may extract the most of free CT and have low influence on the amount of the bound CT including PNBCT and ICBCT in plant samples. The protein noncovalent bound CT (PNBCT) are released from the CT-protein precipitates such as SDS (Makkar, 1989; Stern et al., 1996; Hagerman et al., 1998; Chen and Hagerman, 2004), which was used to determined the protein bound condensed tannins in plant sample. The BCT in the residue remaining from the extraction of ECT and PNBCT is irreversible covalent bound condensed tannins (ICBCT), which is determined directly on the residue and was called fibre bound condensed tannins previously (Jackson and Barry, 1996; Dalzell and Shelton, 1997; Terrill et al., 1998; Mupangwa et al., 2000; Palmer et al., 2000; Lin et al., 2006; 2007).

The interesting result from this study was the binding process of free CT during deteriorative reactions. The free CT forms complexes with protein through noncovalent bonding including hydrogen bonding and hydrophobic bonding (Haslam, 1986; Tiaks et al., 1989; Gallet et al., 1999; Maie et al., 2008). The recalcitrant tannin-protein complexes formed in the senescent pericarps, but having low level (0.12%). PNBCT increased rapidly with a sigmoid model during deteriorative reactions. PNBCT in living hypocotyls were not detected. PNBCT of hypocotyls during storage occurred at the beginning of viability losing, following a sigmoid model as well. This cumulating of PNBCT in hypocotyls was tightly related to survival rate and viability of the hypocotyls (Figure 13). The binding process of free CT with protein corresponds to the viability losing from plant tissues and deteriorative reactions. There was rapid loss of total phenolics and ECT from pericarps during the

first 9 days dry storage. Bernhare-Reversat et al. (2003) observed that extractable phenolics and tannins rapidly lost from eucalypt leaf litter. The result from this study was that the rapid disappearance of total phenolics and condensed tannins was partly combined with protein and formed resistant complexes in drying pericarps. At the 9 days of storage, the disappearance of ECT could be ascribed to this transformation. The remainder disappearance of extractable total phenolics and ECT suggested that other chemical process could be involved in phenolics and CT disappearance along with microbial processes, including photo-oxidize, polymerize and irreversible covalent linking with other macromolecules. Although the slight increase of ICBCT was observed at 27 days, the tannins might be present but were not totally detected because of the limitation of the butanol/HCl assay for ICBCT (Makkar et al., 1999). Almost the disappearance of the ECT formed complexes with protein and the TCT did not decrease at the end of 27 days storage for hypocotyls inconsistent with the large disappearance in pericarps. It is interesting to note that there are few ECT combining with protein in culture hypocotyls, as live plant tissues.

The MALDI-TOF MS of ECT from dried hypocotyls of *A. corniculatum* showed that the chemical structure properties were significantly influenced by dry storage. It could be concluded from these results that the chemical structure of ECT extracted from the dried hypocotyls by 70% acetone solution was significant different from the fresh hypocotyls. The MALDI-TOF spectra of ECT from the fresh and dried hypocotyls showed that the pattern of hydroxyl and distribution of the oligomers with relatively lower DP changed obviously. These results might be caused by the following facts. First, the oligomers with more hydroxyl possess stronger protein binding capacity than other oligomers or polymers in ECT, and CT of the shorter chain length would possess more chances to bind to proteins due to their small molecular size and fine solubility. Secondly, the biomacromolecule, mostly including proteins and fibre, would be selective binding those flavan-3-ol oligomers with more hydroxyl and relatively lower DP in plant tissues with deteriorative reactions during dry storage.

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