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Original article

Estimation of Nuclear DNA Content in Tannin-rich Medicinal Plant *Cornus officinalis* by Flow Cytometry

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ABSTRACT

Objective The amount of nuclear DNA (C-value) is a key biodiversity character that provides strong unifying elements in revealing the phylogenetic regularity and relationship between genome size and functional traits for plant resource. The estimation of C-values could primarily extend our knowledge on the genetic background and genome diversity for medicinal plants, and thereby the variation of pharmacological constituents and phylogenetic mechanism of medicinal plant taxa will be revealed. However, a large number of medicinal plants (e.g. *Cornus officinalis*) typically contain a series of secondary metabolites, especially tannic acid, which would significantly affect the estimation of DNA content by flow cytometry (FCM). Methodological discussions and improvement need to be made to solve this problem. **Methods** Two isolation buffers LB01 and Otto I were selected to prepare nuclear suspension with additional treatments of pre-soaking and centrifugation combination of gradient centrifugal force and duration. The best isolation and estimation methods were determined by FCM measurement in *C. officinalis*. **Results** The dry leaves were pre-soaked in Otto I buffer for 15 min and the Otto I nuclear suspension was centrifugated at 1.0×10^3 *g* for 2 min. The results showed that debris and nuclei were better separated and the scatterplots of good quality were obtained with low coefficient of variation (CV). Contrarily, the nuclear DNA content of *C. officinalis* could not be accurately estimated for nuclei extracted by LB01 buffer. Finally, 2C-value and genome size of *C. officinalis* were first estimated as 5.92 pg and 2893 Mbp, respectively. **Conclusion** The new methods proposed here are able to accurately estimate DNA content of *C. officinalis*, which provides valuable references for the estimation of genome size in other tannin-rich medicinal plants.

Key words

Cornus officinalis; C-value; DNA content; flow cytometry; genome size; tannic acid

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1. Introduction

The amount of nuclear DNA (C-value) is an important biodiversity character that reflects the relationships between genome size and diverse characters at many levels ranging from the nucleus and cell to tissues and organisms. For holoploid genome, the abbreviations of C-value are given with numerical prefix such as 1C-, 2C-values and so on (Bennett, 1987; Bennett and Leitch, 2005). To date, researchers have estimated C-values for 8509 plant species as reported in the most recent Plant DNA C-values Database (Release 6.0): http://data.kew.org/cvalues/ (Bennett and Leitch, 2012), and the electronic databases thereby provided a powerful global research platform for biological scientists in the world. Particularly, continued studies have confirmed that genome size could be associated with other functional traits such as nuclear and cell size, seed mass, plant growth form, distribution, specific leaf area, photosynthetic rate, growth rate, and/or cell- and life-cycle length (Beaulieu et al, 2007a; 2007b; 2008; 2010; Hodgson et al, 2010; Leitch et al, 2005; 2009; Ohri, 2005). Such growing findings also raised interesting questions that how much variations there were in nuclear DNA content and how the C-values, phenotype (e.g. pharmacological constituents), and phylogenetic relationships in the same genus or total family for medicinal plants co-varied. The estimation of nuclear DNA content could play a leading part in the research on the genetic background and genome diversity for medicinal plants. Furthermore, increasing accessibility and ease of access to the growing nuclear DNA amounts would be made available to reveal the variation of pharmacological constituents and phylogenetic mechanism of medicinal plant taxa.

Flow cytometry (FCM) is a powerful technique for sorting and analyzing cells, nuclei, and chromosomes efficiently and accurately. Since Galbraith et al (1983) used FCM to successfully estimate the nuclear DNA content of 17 species for the first time, this method has been widely used for plant nuclear DNA content (C-value) measurement and ploidy screening (Doležel and Bartos, 2005), cell cycle assessment (Lee et al, 1996), karyotype analysis (Doležel et al, 1994), chromosome sorting (Doležel et al, 2011), and chromosome library construction (Arumuganathan et al, 1994). Tannic acid (TA) is known as the most significant compound of plant secondary metabolites that can interfere with Galbraith's, LB01, and Tris·$MgCl_2$ isolation buffers in FCM measurement (Greilhuber et al, 2007). Loureiro et al (2006) termed the involvement of TA in the estimation of nuclear DNA content as "TA effect", indicating an extensive phenomenon that the secondary metabolite TA in plants could cause the increased side scatter and its coefficient of variation in FCM measurement. Due to the presence of such negative effects, the accumulation of nuclei and debris particles were difficult to be separated. Consequently, it remained problematic to measure nuclear DNA content in tannin-rich plants (Doležel et al, 2007). As for medicinal plants, specifically the tannin-rich populations, few methodological discussions and investigations have been reported in this field on account of the complexity of the chemical composition. Over the years, growing interests regarding C-value of medicinal plants and its key role in the phylogeny and evolution of medicinal plants have been recognized. Therefore, it is essential to identify and propose a set of mature and reliable methods on the estimation of C-value of tannin-rich medicinal plants. *Cornus officinalis* Sieb. et Zucc., which is classified into genus *Cornus* L. (Cornaceae), is one of the popular Chinese materia medica (CMM) with the important medicinal values. *C. officinalis* has complex chemical composition, and so far the major constituents isolated are TA (tannins), sugars, organic acids and their esters, iridoids, and other components (Cheng, 2011). As *C. officinalis* had abundant tannins in its tissues and organs, we took *C. officinalis* as the tannin-rich model to optimize the methods of estimating C-value of tannin-rich medicinal plants and thus aimed at providing both theoretical and methodological references for the research in this field.

2. Materials and methods

2.1 Preparation of sample plant material

Fresh young leaves of *C. officinalis* (identified by Dr. Cheng-ke Bai, Shaanxi Normal University (specimen collection number: FP001, deposited in the specimen herbarium of College of Life Sciences, SNNU), grown in C. *officinalis* germplasm garden in Shaanxi Normal University, were collected 30 min before chopping. For dry leaves, fresh young leaves were collected, immediately dried with silica gel for about one week and stored in –20 °C.

2.2 Preparation of standard plant material

According to Bai et al (2012), *Pisum sativum* L. and *Vicia faba* L. were selected as standard plants in this experiment. The seeds kindly provided by Dr. Jin-feng Gao in College of Agriculture of Northwest Agriculture and Forestry University were soaked and sowed in tubs filled with nutritive soil, and were grown at room temperature. Fresh young leaves of standard plants were collected with sealed bags 30 min before chopping and were cryopreserved for use.

2.3 Preparation of isolation buffer and nuclear suspension

LB01 and Otto's buffers were prepared using the method suggested by Doležel et al (2007). According to Bai et al (2012), 2 μL/mL β-mercaptoethanol was added to LB01 buffer. In preliminary experiments, we found that the nuclear DNA content of *C. officinalis* could not be accurately estimated by completely referring to the procedure and methods suggested by Doležel et al (2007). Therefore, extraction procedures and methods were optimized on the basis of preliminary experiments. For fresh young leaves, the nuclei were extracted with LB01 one-step and Otto's two-steps by quickly and vertically chopping the young leaves; For silica gel-dried materials, a treatment of pre-soaking for approximately 15 min in Otto 1 buffer was carried out before quickly and vertically

chopping the dry leaves. LB01 buffer was filtered through 30 μm filter (Celltrics®, Partec) and stored for use. Nuclear suspension extracted by Otto 1 was centrifuged at 4 °C under a set of combinations of gradient centrifugal force (1.0×10^3 *g*, 2.0×10^3 *g*, and 3.0×10^3 *g*) and duration of centrifugation (1, 2, and 5 min) (dry leaves + Otto 1 + 15 min soaking + *V. faba* + 1.0×10^3 *g* for 1 min; dry leaves + 15 min soaking + Otto 1 + *V. faba* + 1.0×10^3 *g* for 2 min; dry leaves + 15 min soaking + Otto 1 + *V. faba* + 2.0×10^3 *g* for 2 min; dry leaves + 15 min soaking + Otto 1 + *V. faba* + 3.0×10^3 *g* for 2 min; dry leaves + 15 min soaking + Otto 1 + *V. faba* + 3.0×10^3 *g* for 5 min). After centrifugation, the supernatant in Otto 1 was carefully removed and added Otto 2 solution into the pelleted nuclei to 500 μL. Approximately 15 min prior to FCM measurement, each tube of nuclear suspension was added with 20 μL RNase stock solution (1 mg/mL) and 20 μL propidium iodide (PI) stock solution (1 mg/mL) both at the final concentration of 0.05 mg/mL, and then kept on broken ice in dark until analysis. In the above procedure of extraction and staining, special emphasis should be focused on that the blade was sharp enough to quickly and vertically chop the materials, the chopping operation was kept under low temperature, and the PI dye solution and dyed nuclear suspensions were stored in dark.

2.4 FCM measurement and statistical analysis

In order to prevent nuclear adhesions, the nuclei suspensions were oscillated for 5 s after staining and before FCM measurement, and then were analyzed in Millipore Guava PCA Cytometry. The voltage of PM1 channel was set at 350 V, and for each sample at least 10 000−20 000 cells (nuclei, including debris) were collected. The data obtained by FCM were analyzed using FCM professional software FCS Express 4 Plus (Ormerod, 2008). Scatterplots were used for the evaluation and analysis of the measurement results, and histograms were used for the observation and statistics of cell number. Nuclear DNA content was calculated as follows (Greilhuber et al, 2007):

Sample 2C value = standard 2C value × (sample 2C mean peak position / standard 2C mean peak position)

For the C-value obtained from the above formula, CV (standard deviation divided by the average number of channels) ≥ 5% indicates that the extracted nuclei are not concentrated, which would lead to large deviation of C-value, and samples should be prepared for re-estimation; CV < 5% suggests that the result is correct (Ormerod, 2008). Each sample was measured at least for three repeats, and then the average value was taken. Genome size was calculated according to 1 pg = 978 Mbp in reference (Cavalier-Smith, 1985; Bennett et al, 2000; Zonneveld et al, 2005).

3. Results

3.1 Selection of isolation buffers

The selection of isolation buffers is a critical step in the estimation of plant C-value. Since the additive substance β-mercaptoethanol which showed the characteristics of modulating the action of TA was not included in Galbraith's and Tris·$MgCl_2$ buffers, we used the two most common isolation buffers LB01 and Otto's to extract nuclei and then took comparative analysis in this study. The result showed that no obvious nuclear aggregation particle was observed in FCM scatterplots of nuclear suspension extracted by LB01 buffer from the young leaves. Clear nuclear aggregation particles using *P. sativum* as the internal standard (IS) were found in scatterplots of nuclei extracted from the young leaves by Otto's buffer, although that of *C. officinalis* was not present yet. By LB01 buffer, no obvious nuclear aggregation particle of *C. officinalis* was found in young and dry leaves and no obvious nuclear aggregation particle of *P. sativum* was found in standard material. By Otto's buffer, nuclear aggregation particle of *C. officinalis* was isolated with less and large number in young and dry leaves respectively, and that of *P. sativum* was isolated from standard material (Figures 1 and 2). Comparative analysis of preliminary results derived from the two solutions confirmed that TA has more significant influence on LB01 buffer than on Otto's buffer. Therefore, the Otto's buffer was used to extract the nuclei in further experiments.

3.2 Selection of material

Fresh young leaves and dry leaves of *C. officinalis* were used to extract nuclei with Otto's buffer. The results indicated that no nuclear aggregation particles of *C. officinalis* appeared in the results of either young leaves or dry leaves, but considerable difference on that of the IS *P. sativum* existed between the two kinds of materials, exhibiting that the nuclear number of *P. sativum* in the young leaves was apparently less than that in the dry leaves, and the debris particles were not well separated (Figure 2). These results elucidated that the standard was affected by TA in the young leaves of *C. officinalis*, and thus leading to more dispersed nuclei. Also, these results implied that using dry material to extract nuclei with Otto's two-steps would probably obtain better FCM results. As a result, dry leaves were employed for further experiments.

3.3 Selection of standard plant

Preliminary results using *P. sativum* as IS and Otto's as isolation buffer showed that part overlap appeared in the nuclear particles between *C. officinalis* and *P. sativum*. This observation revealed that the two species were adjacent in nuclear DNA content, and it was difficult to separate the two nuclear particles in the longitudinal coordinates of low resolution. As *V. faba* was used as IS, clear nuclear aggregation particles of the standard and sample were separated. Therefore, *V. faba* was selected as IS to estimate C-value in the next process. The nuclear DNA content of *V. faba* was accurately determined in this study, *Oryza sativa* L. subsp. *japonica* var. *nippobare* with accurately sequenced C-value (2C = 0.91 pg) was selected as the IS to estimate the 2C value of *V. faba*, which was estimated as accurate value of 26.57 pg (Figure 3).

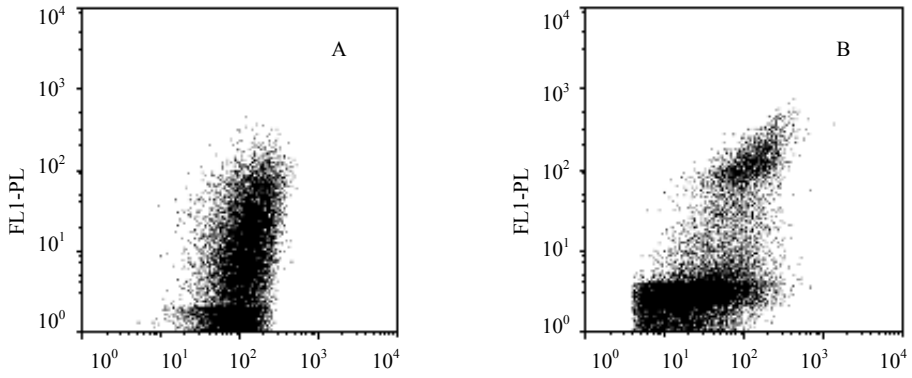

Figure 1 FCM scatterplots of nuclei in young leaves of *C. officinalis* extracted by LB01 (A) and Otto's (B) buffers

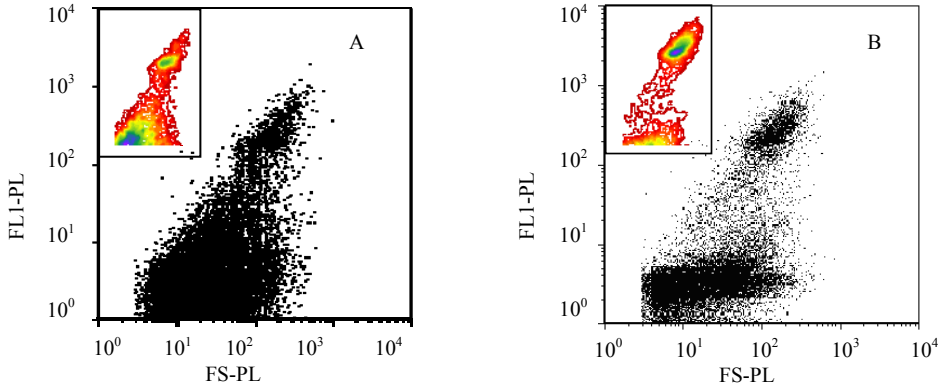

Figure 2 FCM scatterplots and contour of nuclei extracted by Otto's buffer from young leaves (A) and dry leaves (B) of *C. officinalis*

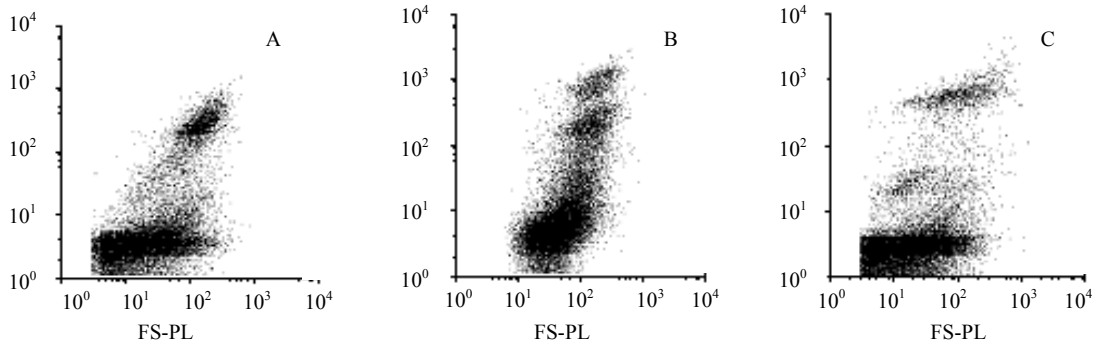

Figure 3 FCM estimation of *C. officinalis* using *P. sativum* (A) and *V. faba* (B) as IS and scatterplot of *V. faba* (C) using *O. sativa*

3.4 Pre-soaking of dry leaves

In preliminary experiments, we found that nuclear suspensions of high quality could not be obtained by centrifugation and filtration after quickly and vertically chopping the silica gel-dried material directly in Otto 1 buffer. Although the nuclear concentration particles were visible, the amount of nuclei was rather less. Nonetheless, clear nuclear aggregation particles with more nuclei were observed in nuclear suspensions with additional treatment of soaking the dry leaves in Otto 1 buffer for approximate 15 min before chopping in 4 °C Otto 1 buffer (Figure 4). This result indicated that the influence of "TA effect" that could affect both the standard and the samples was partly eliminated in dry materials by pre-soaking in Otto 1 buffer. Therefore, it was suggested that the treatment of pre-soaking could effectively reduce or eliminate the effect of TA, probably by promoting cell dehydration and subsequent absorbent of water to achieve the separation of nuclear and cytoplasm.

3.5 Effect of centrifugation on FCM measurement

The above results showed that FCM scatterplots of better quality could be obtained by using Otto's buffer to extract nuclei from the dry leaves that were pre-soaked for 15 min. To further investigate the phenomenon that nuclei and debris were difficult to separate for the occurrence of TA effect, additional steps of the combination of gradient centrifugal force and duration of centrifugation were performed after

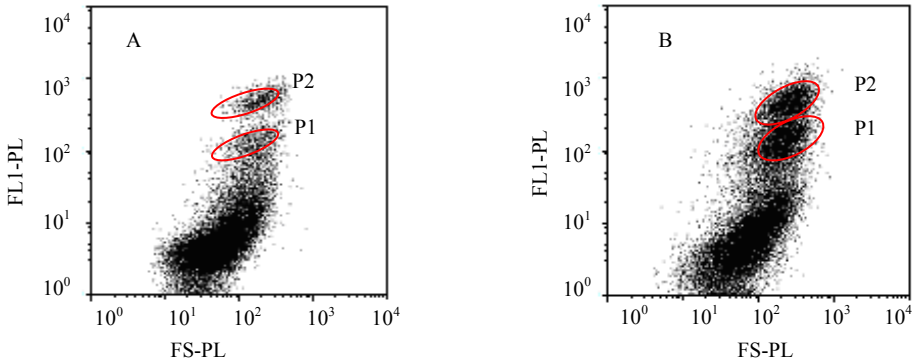

Figure 4 FCM scatterplot of estimation results using un-soaked (A) and soaked (B, for 15 min) dry leaves
P1: *C. officinalis*; P2: *V. faba*

nuclear suspensions were prepared by Otto's buffer. After centrifuged at 1.0×10^3 *g* for 1 min, nuclear aggregation particles of *C. officinalis* and *V. faba* were gathered with debris and were difficult to separate, which might due to less centrifugation strength that led to weak separation of nuclei and debris. More concentrated nuclei of the IS and the sample in larger amounts were observed in the combination of centrifugation at 1.0×10^3 *g* for 2 min with the additional step of pre-soaking, indicating that the treatment of centrifugation at this level could better dissociate nuclei and be propitious for taking the way to improve the accuracy of FCM measurement. Lower or higher capacity of centrifugation treatment led to weak distribution of particles in scatterplots and histograms (Figure 5). According to these results, it was therefore inferred that nuclei appeared lysed with the increase of centrifugation strength, resulting in the increasing CV. It was also demonstrated that the combination of centrifugation at 1.0×10^3 *g* for 2 min had ideal effect on the nuclear separation of *C. officinalis* and *V. faba*, which could significantly inhibit the effect of TA. As a result, 2C-value and genome size of *C. officinalis* were estimated as 5.92 pg and 2893 Mbp, respectively (Table 1).

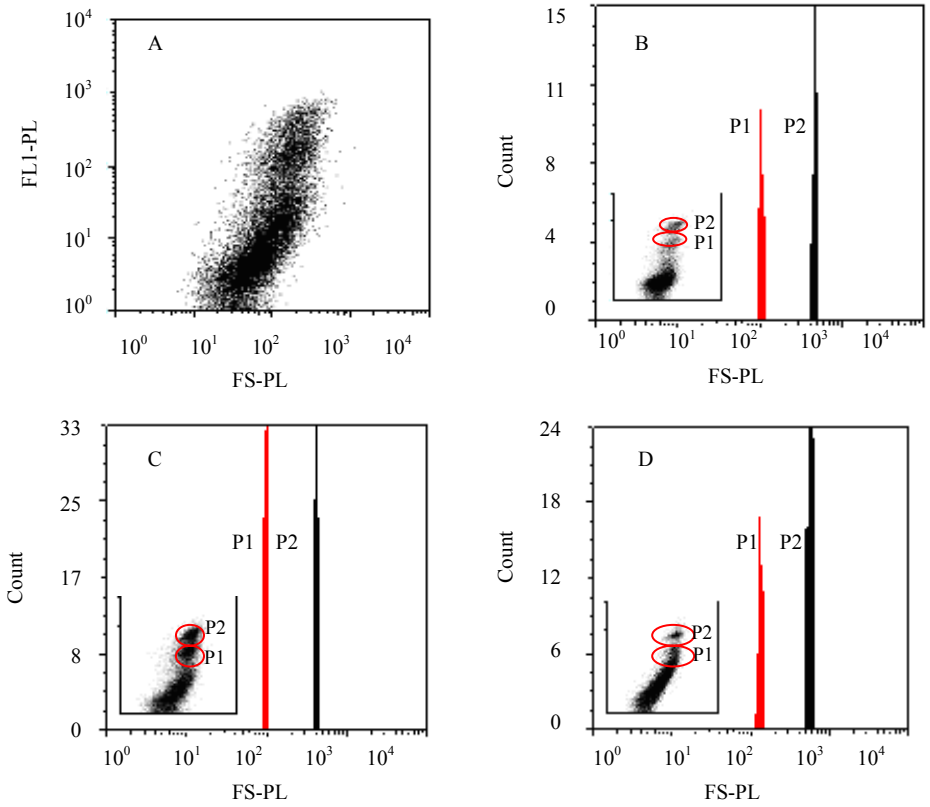

Figure 5 Scatterplots and histograms of estimation of *C. officinalis* by FCM at different centrifugation levels
A: 15 min soaking + 1.0×10^3 *g* for 1 min; B: dry leaves + 1.0×10^3 *g* for 2 min; C: 15 min soaking + 1.0×10^3 *g* for 2 min; D: 15 min soaking + 3.0×10^3 *g* for 2 min; P1: *C. officinalis*; P2: *V. faba*

Table 1 Estimation of C-value of *C. officinalis* at different centrifugation levels

Experimental groups	2C-value / pg	CV / %	Genome size / Mbp	Comments
dry leaves + 15min soak + Otto 1 + *V. faba* +1.0×10^3 *g* for 1 min	−	−	−	Nuclear aggregation particles were gathered with debris and could not be separated.
dry leaves + Otto 1 + *V. faba* + 1.0 × 10^3 *g* for 2 min	6.03 ± 0.11	4.43	2946	Nuclear aggregation particles and debris particles were obviously separated, with less amounts of nuclei.
dry leaves + 15 min soaking + Otto 1 + *V. faba* + 1.0 × 10^3 *g* for 2 min	5.92 ± 0.11	3.10	2893	Nuclear aggregation particles of *C. officinalis* and *V. faba* were well separated, with more nuclei and less CV, 2C values were stable among each repeat.
dry leaves + 15 min soaking + Otto 1 + *V. faba* + 3.0 × 10^3 *g* for 2 min	6.09 ± 0.19	5.90	2978	Nuclear aggregation particles of *C. officinalis* and *V. faba* could be acceptably separated, with CV beyond 5%, 2C values were not stable among each repeat.

4. Discussion

The secondary metabolites in plants could affect nuclear extraction and PI staining in the estimation of plant nuclear DNA content by FCM measurement. Currently, such components are identified as phenolic substances possessing active hydroxyl groups and providing free electrons capable of forming hydrogen bonds, which include glycosylated or non-glycosylated monomers (e.g. anthocyans and flavonoids), oligomers, and polymers. The most widely known component is TA (Greilhuber, 1986; Noirot et al, 2000; Price et al, 2000). TA, also known as tannins, is a class of complex compounds that can be divided into condensable tannins and hydrolyzable tannins. Previous studies have clarified that TA could change the side scatter properties to induce interferential nuclear and non-nuclear aggregation, and thus affect the accurate measurement of nuclear DNA content (Loureiro et al, 2006). At present, fluorescence inhibitors and nuclei adhered with debris (coatings of debris) were considered probably responsible for the interference of the above-mentioned aggregations, which could cause two additional effects of fluorochrome binding and debris coating (Doležel and Bartos, 2005; Greilhuber et al, 2007). As one of the CMM, *C. officinalis* contained considerable amount of TA, which made it difficult to accurately estimate nuclear DNA content. The negative effect of TA typically led to failure of clearly separating the nuclear aggregation particles of the IS and the sample, which also reflects the "bottleneck" problem on the estimation of nuclear DNA content in medicinal plants (especially tannin-rich plants).

In view of this, we adopted the tannin-rich plant *C. officinalis* as TA rich model plant to optimize the estimation methods of C-value of medicinal plants based on previous preliminary experiments. The treatment group of Otto's buffer, dry leaf tissues with pre-soaking treatment and centrifugation at 1.0 × 10^3 *g* for 2 min was determined as the best combination. This result supported the result that LB01 buffer was more affected than Otto's buffer by TA (Loureiro et al, 2006), showing that nuclei concentration particles were not separated in nuclei suspensions prepared by LB01 isolation buffer. In general, the different components of buffers were responsible for the extraction quality. Otto's buffer exhibited less significant "TA effect", probably due to the higher concentration of Triton X-100, which could disperse chloroplast and reduce the aggregation of nuclear and cytoplasmic debris (Doležel and Bartos, 2005). Since the selection of experimental materials is also an important factor, the weak effect of TA in dry leaves may be related to cell dehydration and degradation of cytoplasmic compounds in the drying process. With additional pre-soaking treatment, cells of dry leaves could sufficiently absorb water and then release nuclei easily in the chopping process, which would effectively prevent TA binding to nuclear DNA. In addition, the centrifugation level also has the important effects on estimation results. We have determined that the centrifugation level of 1.0 × 10^3 *g* for 2 min could significantly reduce the TA effect in *C. officinalis*, although it was unable to eliminate the negative effect of cytosol by centrifugation in *Coffea liberica* Bull ex Hiern and *Petunia hybrida* Vilm by Noirot et al (2003; 2005). As a result, the centrifugation strength should be specifically selected according to the characteristics of main chemical components to minimize the negative effects of secondary metabolites that interfered with nuclear extraction and DNA staining in the estimation of nuclear DNA content of medicinal plants. Furthermore, these results in the present study will provide the valuable references for estimating nuclear DNA content of medicinal plants that are rich in TA or other secondary metabolites by FCM.

5. Conclusion

In summary, the present study has demonstrated that the treatment combination of Otto's buffer, pre-soaking dry tissues, and particularly the appropriate centrifugation strength can effectively inhibiting or ameliorating the "TA effect" in C. *officinalis*. The novel procedures of this study (pre-soaking and centrifugation) therefore contribute to solving the problem that C-value couldn't or was unable to accurately be obtained from TA-rich plants primarily through preventing TA affecting the components of nuclei. Furthermore, it was conceivable that the above procedures applied equally to other medicinal plants rich in secondary metabolites. Taking this point into account, the further estimation of C-value of medicinal plants should be focused

on optimizing these factors (procedures) and determining the ideal method in FCM research. These above results have clearly been of value for the systematics, phylogeny, and evolution, as well as the conservation, evaluation, and utilization of the medicinal plants, given the scope and of growing the interests in studies addressing the correlations between genome size and a range of biological characters in the aspects of molecular, cellular, developmental, ecological, and evolutionary levels for medicinal resources.

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