Estimation of Nuclear DNA Contents of Three Economically Important Plant Species by Laser Flow Cytometry

تحديد محتوى الحمض النووي لثلاثة أنواع من النباتات ذات الأهمية الزراعية والأقتصادية بواسطة مقياس التدفق الخلوي الليزري

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Abstract

Nuclear DNA content and genome size are key factors in biology and biodiversity, and have important implications in modern molecular genetic studies. Diesel tree (*Copaifera officinalis* L.) and switchgrass (*Panicum virgatum* L. cv. Alamo) are attractive sources for biofuels production, including biodiesel and cellulosic ethanol. On the other hand, horseweed (*Conyza canadensis* L.) is one of the most agriculturally problematic herbicide-resistant weeds worldwide. The nuclear DNA contents (expressed as 1C values) of these economically significant plants were estimated by a fast and valid method of laser flow cytometry. Intact nuclei were isolated from young leaves or roots, stained with propidium iodide, a fluorescent DNA-staining dye, and then analyzed by a flow cytometer simultaneously with nuclei from reference standard plants of known C-values. The 1C-value of the nuclear DNA content of *C. officinalis* was ca. (1,161 Mbp = 1.19 pg DNA). It is approximately similar to that of oilseed rape and soybean, and is more than eight times

the genome size of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia. The 1C DNA content of switchgrass (*P. virgatum*) cv. 'Alamo' tetraploid ecotype was estimated at 1,445 Mbp (= 1.48 pg DNA), which is more than ten times the genome size of *A. thaliana*. Finally, the DNA content of the diploid horseweed (*C. canadensis*) was ca. (378 Mbp = 0.39 pg DNA), which is approximately 2.8 times the genome size of *A. thaliana*, and smaller than the reference genomes of rice and poplar. These data will be useful for molecular and genomic approaches, such as genome sequencing projects and construction of genomic libraries.

Keywords: flow cytometry, DNA content, genome size, 1C-value, *Copaifera officinalis*, diesel tree, switchgrass, horseweed, *Conyza canadensis*.

ملخص

يعتبر محتوى DNA النووي وحجم الجينوم عوامل رئيسية في علم الأحياء والتنوع البيولوجي، ولها آثارهامة في الدراسات الجينية الجزيئية الحديثة. شجرة الديزل (*Copaifera officinalis* L.) ونبات switchgrass (*Panicum virgatum* L.) من سُلالة ألامو من المصادر الجذابة لإنتاج الوقود الحيوي، بما في ذلك وقود الديزل الحيوي والايثانول المنتج من السليولوز. ومن ناحية أخرى، يعتبر نبات horseweed (*Conyza canadensis* L.) واحدا من أكثرالأعشاب إشكالية في العالم لمقاومته للمبيدات الزراعية. في هذا البحث قدرت محتويات DNA النووية للنباتات المذكورة أعلاه بطريقة سريعة عن طريق مقياس التدفق الخلوي الليزري، بحيث تم عزل الأنوية الخلوية من الأوراق أو الجذور وصبغها بمادة propidium iodide المشع، ومن ثم تم تحليلها من قبل قياس التدفق الخلوي في وقت واحد مع أنوية لنباتات معيارية وذات محتوى DNA معروف أصلا. قدرت قيمة محتوى DNA النووي لشجرة الديزل بحوالي (1,161 Mbp = 1.19 pg DNA) أي أكثر بثمانية أضعاف من حجم جينوم نبات الأرابيدوبسيس *Arabidopsis thaliana* المعياري. وقدر المحتوى النووي لنبات (*P. virgatum*) من سلالة "ألامو" بحوالي (1,445 Mbp = 1.48 pg DNA)، والتي هي أكثر بعشرة أضعاف من حجم الجينوم لنبات *A. thaliana*. وأخيرا، قدر محتوى الحمض النووي للنبات العشبي horseweed بحوالي (378 Mbp = 0.39 pg DNA)، وهو ما يقارب 2.8 أضعاف حجم الجينوم لنبات *A. thaliana*. إن لهذه البيانات فوائد واستخدامات مستقبلية في الأبحاث الجزيئية والجينومية، مثل مشاريع دراسة تسلسل الجينوم وبناء مكتبات الجينوم.

الكلمات المفتاحية: مقياس التدفق الخلوي، محتوى الحمض النووي، حجم الجينوم، شجرة الديزل, *Copaifera officinalis*, switchgrass, *Conyza Canadensis*.

Introduction

The amount of DNA in the un-replicated nucleus of an organism is known as its C-value (Swift, 1950). It stands for the DNA content of the whole chromosome complement or karyotype irrespective of the degree of generative polyploidy of the organism. However, genome size is the DNA content of the monoploid genome or chromosome set (Greilhuber et al., 2005). Nuclear DNA content and genome size are key characters in biology and biodiversity, and have important implications in modern molecular genetics practice (Bennett et al., 2000; Bennett and Leitch, 2005; 2011; Zonneveld et al., 2005). The genome sequencing and chromosomal karyotyping approaches were also based on genome and individual chromosomes sizes. Small genomes and chromosomes were chosen first for DNA sequencing including the model plant *Arabidopsis thaliana* (The *Arabidopsis* Genome Initiative, 2000), and rice that has the smallest genome size among the world's major cereal crops (Sasaki, 1998). In addition, the C-value data have contributed to a variety of related studies such as plant breeding programs, ploidy screening, detection of aneuploids, cell cycle kinetics, and reproductive pathways (Doleˇzel and Bartoˇs, 2005). Moreover, continual improvement of our knowledge of the C-values is important for studying mechanisms in genome size evolution, to probe phylogenetic dimensions among organisms, creating genetic libraries, and as indicators to a broad range of external ecological issues and environmental concerns (Bennett et al., 2000; Bennett and Leitch, 2005; 2011; Zonneveld et al., 2005).

DNA flow cytometry is a fast, valid and relatively cheap method most frequently used to determine nuclear DNA content and ploidy level in many organisms including plants (Marie and Brown, 1993; Bennett et al., 2000; Torrell and Valles, 2001; Doleˇzel and Bartoˇs, 2005; Doleˇzel et al., 2007). In principle, the method involves preparation of suspensions of intact nuclei whose DNA is stained using a DNA fluorochrome such as propidium iodide. The stained nuclei are then classified according to their relative fluorescence intensity or/and DNA content (Doleˇzel and Bartoˇs, 2005).

The diesel tree (*Copaifera officinalis* L.)

Copaifera officinalis, also called ''diesel tree", is known for oleoresin production which results from tapping the trunk of a mature tree (Joyce et al., 2012). The chemical composition of the *C. officinalis* oleoresin is mainly composed of sesquiterpene terpenoids (Chen et al., 2009), which have been suggested to play a role in plant defense against pests and pathogens (Langenheim et al., 1986). In addition, *Copaifera* oleoresin hydrocarbons can be directly used as biofuel in a diesel engine (Calvin, 1980). However, the geographical distribution of *Copaifera* trees is limited to the tropics. Thus, genomics research is crucially required to characterize and exploit the genes involved in *Copaifera* oleoresin biosynthetic pathways. Subsequently, key genes and gene regulation networks could be engineered into potential oilseed plants suitable for temperate regions to complement and increase their bioproducts and biofuel production. Estimation of the nuclear DNA content for the genus *Copaifera* might be useful for such genomics studies, future genome sequencing projects and construction of genomic libraries for the diesel tree. Thus, it was considered for the first time in the present study.

Switchgrass (*Panicum virgatum* L. cv. Alamo)

Switchgrass (*Panicum virgatum* L.) is a perennial grass native to North America. It has been the target for intensive agronomic and breeding research, and biotechnology approaches since it was selected as a model herbaceous biofuel feedstock crop by the United States Department of Energy (US-DOE) (McLaughlin and Kszos, 2005). Switchgrass is a genetically and morphologically diverse species with multiple ploidy levels and ecotypes. Based on flow cytometric studies, most switchgrass cultivars are categorized into two major ecotypes: Lowland ecotypes are primarily tetraploids (2n = 4x = 36), while upland ecotypes are predominantly octaploids (2n = 8x = 72) (Gunter et al., 1996; Hopkins et al., 1996; Narasimhamoorthy et al., 2008). 'Alamo' is a lowland tetraploid switchgrass variety (Hopkins et al., 1996). This cultivar is extensively distributed throughout switchgrass breeding programs in the southern United States and is a parent of several mapping populations (Casler et al., 2011). The current

whole-genome sequencing effort is focused on a high-yielding, 'Alamo' clone, AP13 (http://www.phytozome.net/panicumvirgatum_er.php). Switchgrass genomic resources will accelerate the ability of plant breeders to enhance biomass productivity, reduction of recalcitrance towards better cellulosic ethanol production, better pest resistance, and nutritional quality, particularly for this high-yielding and embryogenic 'Alamo' cultivar. It has been the most-often transformed cultivar that has also been used for intensive cellular and molecular genetic studies during the last decade (Mazarei et al., 2008; 2011; Mann et al., 2009; Nageswara-Rao et al., 2013a,b; Shen et al., 2013).

Using flow cytometry, the nuclear DNA content has been determined for several switchgrass populations of various ploidy levels, including tetraploid ecotypes (Hopkins et al., 1996; Lu et al., 1998; Costich, 2010). However, the nuclear DNA content of this specific ecotype is not well characterized yet, and thus was considered in this study.

Horseweed (*Conyza canadensis* L.)

Horseweed, is a worldwide problematic weed that shares many weediness features with the world's most damaging weeds (Basu et al., 2004; Chao et al., 2005). It has evolved resistance to four herbicide classes in thirteen countries including the United States (Weaver, 2001; Okada et al., 2013; Heap, 2014). In fact, horseweed was the first broadleaf weed to evolve resistance to glyphosate, the most widely used, low cost, highly efficient herbicide for controlling weeds. This intensive use of glyphosate resulted in the emergence of independent resistant biotypes of horseweed in many locations in the United States (Yuan et al., 2010). In addition, a horseweed plant transformation and regeneration method has been developed (Halfhill et al., 2007) that allows for overexpression or knockdown analysis of potential gene targets. Therefore, it is an attractive model for weed genomics approaches that are critical for better understanding of weed biology, weediness molecular genetics, which are crucial for better weed management (Stewart et al., 2009; Peng et al., 2010; 2014; Yuan et al., 2010). In this current study, the genome size of a glyphosate-resistant horseweed accession from Tennessee, USA is determined.

The overall objectives of this research were to estimate the nuclear DNA contents of three economically significant plants species using the technology of flow cytometry, including switchgrass cv. 'Alamo' and the diesel tree as potential bioenergy crops for ethanol and biodiesel production, and horseweed as a key problematic agricultural glyphosate-resistant weed in USA.

Methods

Plant material

The flow cytometric estimations of nuclear DNA contents were achieved through 4-6 replicated measurements from young healthy leaf or root tissues of the plant of interest and the internal standard plant species of known DNA contents (Table 1; see flow cytometric analysis). Preliminary experiments (data not shown) determined the optimal internal plant reference(s) for each tested plant species, and the type of tissue(s) appropriate for subsequent cytometric analyses.

The diesel tree (*C. officinalis*) seeds were kindly provided by James Ackerman at the University of Puerto Rico. Seeds were surface sterilized in 70% ethanol for 2 min, then in 10% household bleach (Clorox, 6 % sodium hypochlorite) for 10 min, followed by washing three times with sterile distilled water. For seed germination, the seeds were placed on sterile filter paper soaked with sterile distilled water in sterile Petri-dishes and incubated under standard growth chamber conditions. About two-weeks old seedling roots were used for the subsequent DNA measurements.

For soybean, oilseed rape, switchgrass, tobacco, horseweed, and rice plants, the seeds were sown in pots, and plants were grown in a greenhouse. Young healthy leaves were used for each measurement (Table 1).

Nuclei Isolation and Staining

For preparation of suspensions of nuclei, approximately 300-500 mg of fresh root tips or young leaf tissues of the experimental plant species and those from the internal reference plant species were co-chopped at

the same time with razor blades in 3-ml ice-cold propidium iodide nuclei staining buffer in a glass Petri-dish, and analyzed simultaneously through the flow cytometer according to the protocol described by Galbraith et al, (1983). In parallel, intact nuclei were isolated and stained with propidium iodide using the *CyStain PI absolute P*-Partec® kit, N.J., USA, according to the manufacturer's instructions. Both procedures were optimized with the following beneficial modifications: 1% (w/v) polyvinylpyrrolidone (PVP-40; Sigma) were added to the extraction buffer to remove phenolic impurities and cytoplasmic compounds from plant nuclei, making the suspension more suitable for flow cytometry (Lee and Lin, 2005). The PVP-40 reduces the crystalline calcium oxalate and other metabolites that block the fluidics system of the flow cytometer. The nuclei suspension was filtrated using 12 x 75 mm, 5 ml polystrene round bottom test tube, 1400 RCF rating, with a nylon-cell strainer cap designed for flow cytometric applications (BD Biosciences Discovery Labware-BD Falcon™, USA). Before staining the nuclei, their filtrates were centrifuged at 120 g for 10 min at 4ºC as recommended by Lee and Lin, (2005) to increase the nuclei number in the final solution.

Flow cytometric analysis

Flow cytometric data was collected from several runs using a 650-nm DL dichroic filter plus a 625-nm BP band-pass filter with a flow cytometer equipped with an argon-ion laser tuned to 488 nm (EPICS XL flow cytometer, Expo 32 ADC software, Beckman Coulter, Miami, Florida). Doublet discrimination was performed using the peak versus integral two parameter histogram method. Mean fluorescence intensity was calculated using a linear scale from a region encompassing the G0/G1 peak as a non-replicating phase, i.e. before the replicating S-phase, or the replicated G2 phase. The G0-G1 phase is easily distinguished from the G2 phase in histograms by lower fluorescence intensity. Plant species of known 1C-value of the nuclear DNA content were used as internal references, including: Tobacco (*Nicotiana tabacum* L. cv. 'Xanthi') for switchgrass (*P. virgatum* cv. Alamo); soybean (*Glycine max* L. cv. 'Jack') and oilseed rape (*Brassica napus* cv. Westar) for the diesel tree (*C. officinalis*); and rice (*Oryza sativa* L. ssp. Japonica

cv. 'Nipponbare') and poplar (*Populus trichocarpa* - black cotton wood) for horseweed (*C. canadensis*) (Table 1). The unknown nuclear DNA content expressed as 1C-values of the diesel tree and horseweed were computed from standard curves of mean fluorescence intensity measured in this study vs. known 1C-values of standard controls obtained from available published data (Table 1; Figures 1,3). The 1C-value for switchgrass was calculated using the formula: (mean fluorescence of plant nuclei/mean fluorescence of standard nuclei) × 1C-value of standard reference (Costich et al., 2010) (Table 1; Figure 2). The C-values typically measured in terms of mass (as picograms, abbreviated pg), or as the total number of nucleotides (as millions of base pairs = mega base pairs, abbreviated Mbp). One pg of pure DNA equals 978 Mbp (Dolezˇel et al., 2003).

Results and discussion

An organism's complexity is not directly proportional to its genome size, where variation in genome sizes is mainly due to repetitive DNA (Gregory, 2001). Genome size can be defined as the total amount of DNA contained within one copy of a genome. Since many angiosperms undergo polyploidy, the monoploid genome size, is often estimated and analyzed. Flow cytometry is a fast and efficient method for such characterization of the plant nuclear DNA content (Bennett and Leitch, 2011). In the study presented here, the genome sizes of the *Copaifera* diesel tree, tetraploid switchgrass cv. 'Alamo', and horseweed were estimated by flow cytometry using propidium iodide as a fluorescent dye. As described in the Methods section above, the 1C-values of the three plant species under study were calculated using known C-values of standard references from the corresponding published literature. In this paper, we used the most recent available C-values of these internal references (Table 1) compared to our early data shown in a conference abstract (Al-Ahmad et al., 2008). Thus, the variation in the estimated C-values of the same tested plants in our two reports is due to using the most updated C-values of these internal standard references in this present study.

Table (1): Flow cytometric analysis of the nuclear DNA content of the diesel tree, switchgrass and horseweed.

Organism	Species	Source of plant material	Mean of fluorescence intensity*	Coefficient of variation of the G1 peak (%)	1C-value (Mbp)**	1C DNA (pg)	2C DNA (pg)!	Reference for the 1C-value
Soybean	*Glycine max* L. cv. Jack	Young leaves	301.6	3.7	1,115.00	1.14	2.28	Schmutz et al., 2010; Arumuganathan and Earle, 1991
Oilseed rape	*Brassica napus* cv. Westar	seedling leaves	335.1	5.0	1,128.60	1.15	2.31	Johnston et al., 2005
Diesel tree	***Copaifera officinalis* L.**	**Seedling roots**	**414.6**	**5.6**	**1,160.62**	**1.19**	**2.37**	**This study**
Switchgrass	***Panicum virgatum* L. cv. 'Alamo'**	**Young leaves**	**198.8**	**12.2**	**1,444.74**	**1.48‡**	**2.96**	**This study**
Tobacco	*Nicotiana tabacum* L. cv. Xanthi	Young leaves	697.1	5.1	5066.04	5.18	10.36	Leitch et al., 2008; Bennett and Leitch, 2012
Horseweed	***Conyza canadensis* L.**	Young rosette leaves	**374.0**	**14.5**	**377.88**	**0.39**	**0.77**	**This study**
Rice	*Oryza sativa* L. ssp. Japonica cv.Nipponbare	Young leaves	466.2	8.6	420.00	0.43	0.86	Goff et al., 2002
Poplar	*Populus trichocarpa* (Black cotton wood)	Young leaves	608.2	5.3	485.00	0.49	0.99	Tuskan et al., 2006

* Mean of 4-6 readings.

**One pg of DNA equals 978 Mbp (Dolezˇel et al. 2003).

! Nuclear DNA content of a replicated nucleus.

‡The 1C-value of the lowland ecotype of switchgrass (*P. virgatum* L. cv. Alamo) was calculated using the formula: (mean fluorescence of switchgrass nuclei/mean fluorescence of tobacco standard nuclei) × 1C-value of tobacco as a standard reference.

Nuclear DNA content of the diesel tree (*Copaifera officinalis* L.)

Copaifera tropical tree is a promising species for bioproducts and biodiesel production (Joyce et al., 2012). The 1C DNA content of this family ranges from 0.31 pg to 27.40 pg (Bennett and Leitch, 2012). In this study, a flow cytometric analyses of seedling root samples were run and analyzed simultaneously against samples of young leaf tissues from the calibration standard plants of oilseed rape and soybean. The 1C-value representing the haploid genome size of *C. officinalis* was ca. (1,161 Mbp = 1.19 pg DNA) (Tables 1; Figure 1). It is approximately similar to the genome sizes of oilseed rape and soybean, and is more than eight times the genome size of the model plant *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia. (135 Mbp; http://www.arabidopsis.org/portals/genAnno).

Nuclear DNA content of switchgrass (*Panicum virgatum* L.) 'Alamo' tetraploid ecotype

A series of flow cytometric studies have been conducted to estimate the nuclear DNA content and the ploidy level of several switchgrass populations, and were useful in discriminating between tetraploid (2n = 4x) and octaploid (2n = 8x) switchgrass plants (Hopkins et al., 1996; Wullschleger et al., 1996; Lu et al., 1998, Costich et al., 2010). According to the studied switchgrass populations, the average 2C-values were 2.35-3.1 pg for the studied tetraploid populations, and 4.88-6.1 pg for the examined octaploid populations (Hopkins et al., 1996; Lu et al., 1998; Costich et al., 2010; Bennett and Leitch, 2012).

Analyses of nuclei isolated from young stems and stained with propidium iodide indicated that 'Alamo' switchgrass is a tetraploid (Wullschleger et al., 1996). The authors used arbitrary units from limited flow cytometric analyses to describe the DNA content of 'Alamo' cultivar against other switchgrass cultivars with different ploidy levels. From our own flow cytometry experiments it is estimated that 'Alamo' ecotype has a nuclear DNA content of about 1,445 Mbp, which is equivalent to 1.48 pg/haploid genome (thus, the 2C-value = 2.96 pg/

nucleus) (Tables 1; Figure 2) . This value is more than ten times the genome size of *A. thaliana* and lies within the range of previously reported genome sizes of other switchgrass tetraploid cultivars, as described above.

Nuclear DNA content of the economically-important weed (*Conyza canadensis* L.)

Despite their agricultural and economic significance, weeds genomics data are very scarce. Horseweed exerts a global agricultural challenge mainly due to its herbicide resistance. The 1C nuclear DNA content of a Tennessee-accessed glyphosate-resistant horseweed biotype estimated by flow cytometry was ca. (378 Mbp = 0.39 pg DNA), which is smaller than the genomes of rice and poplar tree reference plants (Tables 1; Figure 3). Also, these estimations indicate that the genome size of horseweed is about 2.8 times the genome size of *A. thaliana*. This simplicity of horseweed genome should facilitate both genomics and biotechnology research studies particularly the evolution of herbicide-resistance. On the other hand, these findings have encouraged our colleagues in the laboratory of Prof. Neal Stewart at the University of Tennessee; who built a draft horseweed genome assembly using integrated data from multiple sequencing platforms (Peng et al., 2014). Based on their genomic sequencing data, the horseweed genome size is about 335 Mbp, which is roughly close to our herein-shown data that were obtained earlier by flow cytometric analysis. One of the example of the variation in the genome size measured by flow cytometry and other high-throughput technologies, is that of willow tree (*Salix purpurea*), which was 450 Mb measured by flow cytometry comparing to an early estimation by ALLPATHS that was of 379 Mb (*Salix purpurea* v1.0, DOE-JGI, http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Spurpurea_er). Therefore, the estimation of DNA content through a fast and relatively cheap flow cytometric analysis can provide us with initial valuable knowledge of the genome size of a desired plant species, which could be then subjected to subsequent high-throughput, but time

consuming and high-cost sequencing technologies, for example the next-generation sequencing technology, which is valid for advanced descriptive structural and functional genomics approaches.

Conclusions

The nuclear DNA contents (genome size) of three economically significant plants including: diesel tree (*C. officinalis* = 1,161 Mbp), switchgrass (*P. virgatum* = 1,445 Mbp) cultivar 'Alamo', and horseweed (*C. Canadensis* = 378 Mbp) were estimated by a rapid method of flow cytometry. These data will be a valuable starting points towards many areas of subsequent molecular and genomic research, ranging from evolutionary studies to genome mapping and construction of genomic libraries.

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Competing interests

The authors declare that they have no competing interests.

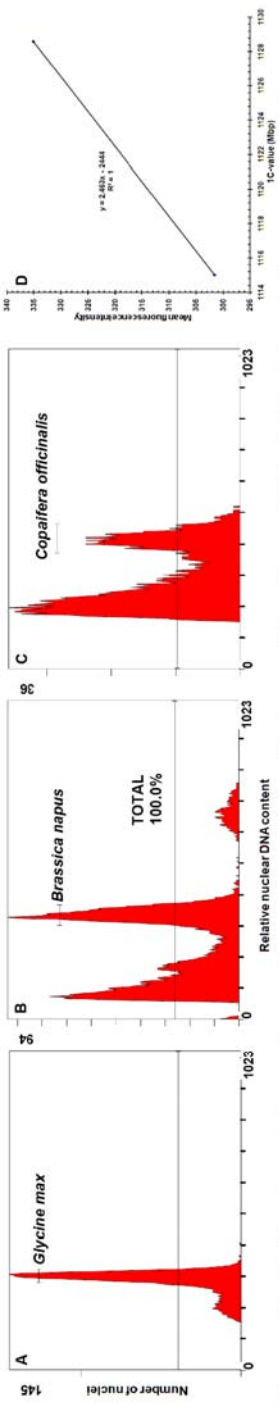

Figure (1): Estimation of the nuclear DNA content of the diesel tree (*Copaifera officinalis* L). Selected flow cytometric histograms obtained after analysis of propidium iodide-stained diesel tree plant nuclei and nuclei from reference standards that were isolated, stained and analyzed simultaneously (Panels **A-C**). **D**. The 1-C-value of the diesel tree (*C. officinalis* L.) was computed using the equation of a linear scale from a region encompassing the G0/G1 peak: (y = 2.463x - 2444). The 1C-values including those of calibration reference standards of soybean (*Glycine max* L. cv. Jack) and oilseed rape (*Brassica napus* cv. Westar) (**Table 1**).

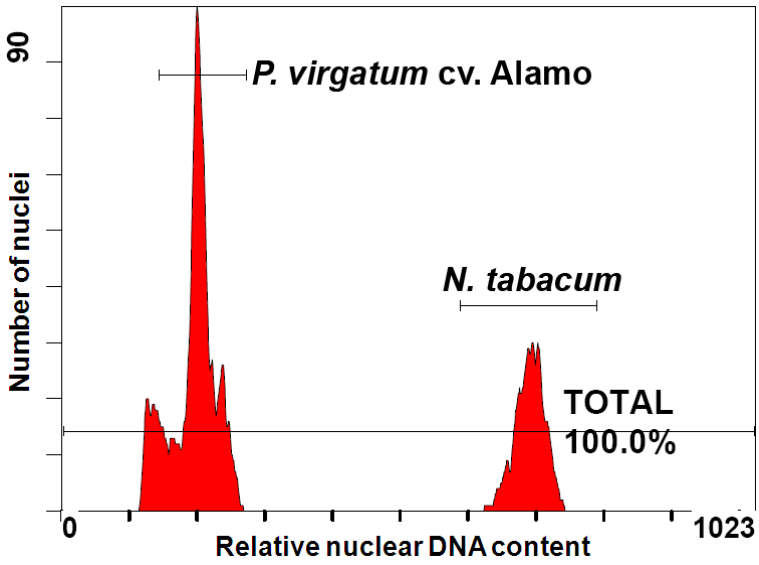

Figure (2): Relative nuclear DNA content of switchgrass (*Panicum virgatum* L.) cv. 'Alamo'. The histograms of DNA content were obtained after flow cytometric analysis of propidium iodide-stained nuclei of switchgrass and tobacco (*Nicotiana tabacum* cv. 'Xanthi') reference standard, which were isolated, stained and analysed simultaneously (**Table 1**). Numbers appear on the y-axis represent the number of stained nuclei detected by the flow cytometer. The x-axis represents the relative nuclear DNA content.

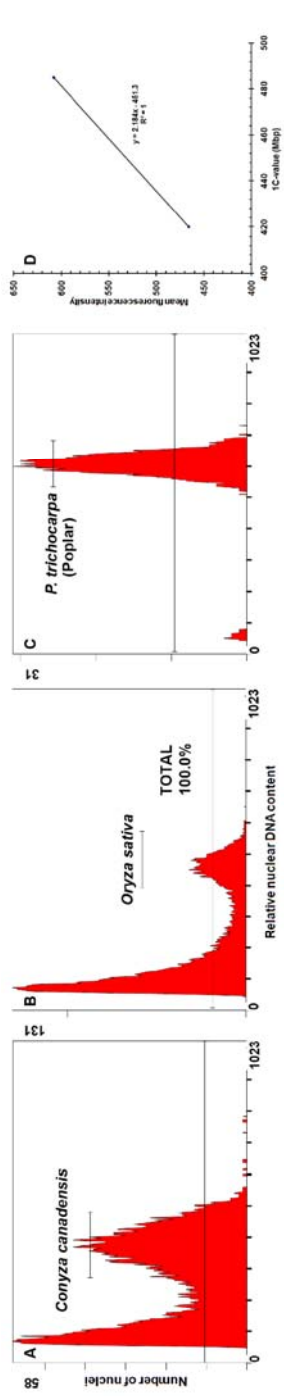

Figure (3): Estimation of nuclear DNA content of horseweed (*Conyza canadensis* L.). Histograms of propidium iodide fluorescence intensity of stained nuclei excited and analyzed by the flow cytometry. Rice (*Oryza sativa* L.) and poplar (*Populus trichocarpa*) served as internal reference standards (**Table 1**) (Panels **A-C**). **D**. The 1-C-value of the horseweed was computed using an equation of a linear scale from a region encompassing the G0/G1 peak: (y = 2.184x - 451.3).

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