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Technique to Obtain Mitotic Chromosomes of *Conyza bonariensis* L. Cronquist (Asteraceae)

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Abstract

Cytogenetic analysis requires cytological preparations that unequivocally reveal the chromosome number and permit optimal visualizations of chromosome morphology for the construction of karyotypes and ideograms. Chromosomal characterization is possible only by establishing these two parameters. To cytogenetically characterize the weed *Conyza bonariensis* (L.) Cronquist (Asteraceae), it was necessary to improve cytological analysis techniques to obtain optimal results. This species belongs to a genus whose plants have wide phenotypic plasticity. These plants can be morphologically differentiated by other types of analysis, and thus the application of this technique will serve as a reference for cytogenetical analysis of other groups of plants that have cytogenetic characteristics similar to those of *C. bonariensis*. The methodology described here highlights three main protocol steps: 1) root tip collection from newly germinated seed radicles and from young root tips of mature plants, 2) pretreatment of meristems with antimitotic agents, both isolated and combined, and 3) acid and enzymatic hydrolytic processes.

Keywords

Hairy Fleabane, Cytogenetics, Chromosomes, Weed

1. Introduction

Cytogenetic analyses require the application of techniques that unequivocally reveal the chromosome number

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and permit optimal visualizations of chromosome morphology for the construction of karyotypes and idiograms. The basic principles for analyses of mitotic and meiotic chromosomes of most plants are similar, consisting of specimen collection, fixation and staining [1].

In mitotic chromosomal analysis, parts of the plant containing meristematic tissue can be used, such as the root [2], buds [3], and also the tissue of callus culture [4]. Regarding meiotic division, anthers of flower buds are used [5].

Several protocols for chromosome analysis are available in the literature; however, it is generally necessary to adapt them to the different plant materials [6]. This is because the same protocol often provides varying results, depending on the plant material on who is applied.

Plants of the *Conyza* Less. genus belong to the Asteraceae family. Plants of this genus, such as *Conyza bonariensis* (L.) Cronquista (native Asteraceae of South America), have caused losses in agriculture due to their invasive capacity and resistance to herbicides such as glyphosate, and recently to ALS herbicides [10]. Herbicide resistance has been reported in Brazil for *C. bonariensis* [7] [8], *C. canadensis* [9] and *C. sumatrensis* [10]. The *Conyza* genus, which includes these species, is known to have wide phenotypic plasticity. The characterization of different morphotypes at the cytogenetic level, in addition to other types of analysis, contributes to the differentiation of these plants.

The first researchers to count chromosomes in *Conyza* ([11]-[13] and [14] [15]) have reported that *C. bonariensis* has the haploid number n = 27, with basic number x = 9, establishing it to have a hexaploid set (2n = 6x = 54).

The karyotype analysis of *C. bonariensis* and four other species of the same genus (*C. blakei*, *C. glandulitecta*, *C. primulaefolia*, *C. sumatrensis* var. *sumatrensis* and *C. sumatrensis* var. *floribunda*) [16] show that all the studied taxa had relatively symmetrical karyotypes with small chromosomes, ranging from 1 to 3 mm in length. Factors such as the small chromosome size, homogeneous morphology of the chromosome, and in several cases, high chromosome number hinder its sufficient spreading in the metaphase plate and its accurate morphologic differentiation.

A protocol that could enhance cytogenetical observations of *Conyza* plants is necessary because, in addition to chromosome size and number, polyploid sets have been reported for some species. Tetraploidy, 2n = 4x = 36 [17]-[20], pentaploidy, 2n = 5x = 45 [20], and hexaploidy, 2n = 6x = 54 [5] [11]-[16] [21]-[23] have previously been reported for *C. bonariensis*. In the mentioned studies above, only one [20] has reported the karyotype of *C. bonariensis*, whereas solely two [18] [16] have reported its cariogram and/or idiogram.

The first step for mitotic chromosomal analysis of any plant sample is to obtain meristematic tissue, because this analysis requires highly proliferative tissue composed of cells that are not functionally differentiated. To date, young root tip meristem is the most used tissue for analysis. Despite its natural high rate of cell proliferation, cell divisions in this tissue are not synchronized in the metaphase stage, when the chromosomes are at their maximum degree of condensation and, therefore, are best visualized for cytogenetic analysis [24].

Obtaining meristem with high yield of metaphase cells will directly depend on the use of substances with antimitotic effect. Antimitotic pretreatment of meristematic tissue, either from radicles, buds, or callus, has the function of depolymerizing the mitotic spindle of the cell, thereby preventing chromosome segregation to the cell poles. Therefore, chromosomes remain in the metaphase plate, increasing the yield of metaphase cells. In addition, antimitotic pretreatment enhances chromosome condensation, evidencing their different constraints and facilitating their morphological identification [25].

As previously mentioned, the antimitotic agent must be applied by a pretreatment of the meristem, under carefully established time and temperature conditions. Among the most commonly used antimitotic agents are colchicine, 8-hydroxyquinoline, α-bromonaphthalene, paradichlorobenzene (PDB), and cold water, all of which block spindle fiber formation [25] [26]. The choice of antimitotic agent is dependent upon the response of each analyzed material.

Following pretreatment, the material requires to be fixed in a solution that preserves the cell and its organelles, without oxidation and degradation. Fixatives generally used in plants include *Carnoy* I (3:1, ethanol:glacial acetic acid) and *Carnoy* II (6:3:1, ethanol:chloroform:glacial acetic acid) [1]. After meristem collection, antimitotic pretreatment and fixation, the material is stored. The fixed sample can be stored under refrigeration for long periods, including years, until they are used for cytological preparations [24].

Slide preparation of the samples begins with the process of softening meristematic tissue by hydrolysis. Softening of the meristematic cell walls is critical to obtain slide preparations without overlapping cells and/or

chromosomes. Hydrolysis may be performed using acid or enzymes, isolated or combined, which act on cell wall. Acid hydrolysis is commonly performed with hydrochloric acid (HCl) at concentrations ranging from 1 N to 5 N, and enzyme hydrolysis commonly uses pectinase and cellulase enzymes.

In this article, we report technical improvements of a cytological preparation protocol for *Conyza* genus, using *C. bonariensis* as a model. This method is expected to serve as a reference for other plants with cytogenetic characteristics similar to those of *C. bonariensis*. Methodological particularities regarding 1) meristem collection from radicles of newly germinated seeds and from young root tips of mature plants, 2) pretreatment of meristems with antimitotic agents, both isolated and combined, and 3) acid and enzymatic hydrolysis are highlighted.

2. Material and Methods

C. bonariensis seeds were collected from uncultivated areas of the city of Campinas, in the state of São Paulo, Brazil, for use in this study. The specie is deposited in the herbarium of the Agronomic Institute of Campinas (IAC 53451).

After collection the seeds were removed from the capitulae and selected using stereoscopic microscopy. Seeds with structural abnormalities or translucent appearance, indicative of the absence of endosperm and/or embryonic tissues, were discarded. The selected seeds were presoaked in distilled water for 48 h at 25˚C ± 2˚C.

2.1. Meristem Collection

Presoaked seeds were divided into three groups according to the time and localization of the collected meristem: 1) seeds presoaked in distilled water for 48 h with meristems collected from the root primordia, 2) seeds newly germinated in Petri dishes, with meristems collected from the radicles, and 3) adult plants grown in greenhouses, with meristems collected from the younger roots.

a) Meristem collection from root primordial

Integuments of presoaked seeds were removed for complete exposure of the embryo using histological needles under a stereoscopic microscope. To avoid loss and/or disintegration of the meristem, the whole embryo was used. At this developmental stage, the cotyledons and the root are closed and translucent. The meristem was sectioned only during cytological preparations.

b) Meristem collection from radicles of newly germinated seeds

Presoaked seeds were uniformly distributed on two sheets of germination paper, moistened with distilled water, and placed in Petri dishes, and they were maintained under artificial light until the emergence of radicles.

Germinated seeds were collected daily, from the first to the fourth day after sowing. To avoid loss and/or disintegration of the root meristem, which is small (≤1 mm), the whole seed was used, *i.e.*, the seed with a developed shoot and radicle. At this stage, the whole seedling must be collected because a portion of it adheres to the germination paper, causing the meristem break. In some situations, adding water until the seedlings are submerged facilitated removal of the meristem. The meristem was excised only during cytological preparations.

c) Meristem collection from young roots of adult plants

Presoaked seeds were sown in two-liter pots containing soil and vermiculite (1:1). The germinated seedlings were kept in a greenhouse until the adult phase.

Root meristems of these plants were collected on two occasions: 1) when the plants were in the vegetative stage, 60 days after sowing (before flowering) and 2) when the plants were in the reproductive phase, 90 days after sowing (after the emergence of the first flower buds). Adult plants were removed from the pots and washed under running water. After this procedure, young root tips of about 4 - 5 mm were cut using scissors. Meristem sectioning for cytological preparations was performed under a stereoscopic microscope.

2.2. Antimitotic Pretreatment

Eleven antimitotic treatments were evaluated for metaphase cell yield. These treatments were based on five antimitotic agents, used isolated or combined. All the treatments were applied for 5 h at 14˚C (**Table 1**).

Pretreated radicles were fixed in *Carnoy* I (3:1, ethanol:glacial acetic acid) and subjected to vacuum for 3 min, allowing rapid penetration of the fixative in the meristem. After fixation, the radicles were stored at room temperature (RT) for approximately 24 h and then stored at −18˚C to −20˚C until the hydrolysis and cytological preparations.

Table 1. Concentrations and combinations of antimitotic agents used in the pretreatment of *Conyza bonariensis* root tips.

	Antimitotic agents
1	Cold water
2	Cycloheximide (6.25 ppm)
3	Colchicine (0.1%)
4	Colchicine (0.5%)
5	Esculin (0.04%)
6	9-Aminoacridine hydrochloride (0.05%) + 8-hydroxyquinoline (0.002 M)
7	9-Aminoacridine hydrochloride (0.05%) + paradichlorobenzene (saturated solution)
8	Hydroxyurea (2.5 mM)
9	Paradichlorobenzene (saturated solution)
10	8-Hydroxyquinoline (0.02 M)
11	8-Hydroxyquinoline (300 ppm) + cycloheximide (6.25 ppm)

2.3. Hydrolysis

Two types of hydrolysis were evaluated in this study: acid hydrolysis and enzymatic hydrolysis. Initially, the radicles were washed with distilled water at RT for 5 min, to remove excess fixative.

Acid hydrolysis was performed at RT with hydrochloric acid at concentrations of 1 N and 5 N. Enzymatic hydrolysis was performed using a mixture of cellulase (2%), pectinase (2%), and hemicellulase (2%) in a humid chamber at 37˚C. HCl hydrolysis was performed for 1 - 15 min, monitored with slide preparations every minute, and enzymatic hydrolysis was performed for 5, 10, 15, 30, 45, and 60 min, monitored with slide preparations every 5 min.

2.4. Cytological Preparations

Four radicles were placed on a slide and sectioned at the area of intersection between the meristematic tissue and the rest of the root using histological needles under a stereoscopic microscope. A drop of 45% acetic acid was added to the material, and the radicles were covered with a coverslip. The assembled slide was heated in the flames of an alcohol lamp and squashed. The coverslip was then removed by immersion in liquid nitrogen, and the exposed material was left to air-dry. After drying, the slides were stained with Giemsa solution (1/100 diluted in Sorensen phosphate buffer) for 10 min [1], air dried, and frozen until use.

The criteria used for treatment evaluation were:

Meristem collection: radicles with whitish tips that probably are an indicative of a goodnumber of meristematic cells for chromosome preparations.

Antimitotic pretreatment: meristem with the highest number of metaphase cells, containing chromosomes with differentiated morphology.

Hydrolysis: Obtaining a good degree of cell squashing and chromosome spreading throughout the slide.

Cytological preparations were analyzed in an Olympus BX50 microscope containing an Olympus model Q-colors3 cooled digital camera. Images were captured using Image Pro Plus version 6.0 software.

3. Results and Discussion

The results of *C. bonariensis* meristem collection are summarized in (**Table 2**) which shows the optimal meristem collection period for obtaining a high yield of metaphase cells, suitable for the cytogenetic analysis of *Conyza*.

Meristems from seeds presoaked in water for 48 h, and from seeds one day after sowing in Petri dishes, showed incomplete breakage of the integument. The meristems were very immature and contained few cell divisions, rendering them unsuitable for cytogenetic analysis.

Table 2. Results of root tip collection from *Conyza bonariensis* L. (Cronquist), from seeds presoaked indistilled water for 48 h at 25˚C ± 2˚C.

Collected meristems		Collection period	Result[1]
Root primordia		Immediately after presoaking	−
Radicles from seeds germinated in Petri dishes		1 day after sowing	−
		2 days after sowing	+
		3 days after sowing	+
		4 days after sowing	+
Radicles from adult plants	Vegetative phase	60 days after sowing (before flowering)	+
	Reproductive phase	90 days after sowing (after emergence of the first flower bud)	+

(−) negative result = very few mitotic cells; (+) positive result = with mitotic cells.

Meristem collection in these periods may have been impracticable because *C. bonariensis* meristem is covered by a membrane. The meristem was adhered to this membrane and, being incipient, compromised the excisions for cytological preparations. No reports of this membrane in *Conyza* radicles were present in the existing literature. A similar membrane in *Bidens segetum* Mart. ex Colla was reported [27]. In this Asteraceae plant, the authors reported that the embryo is internal to the cypsela (fruit) and covered by a membranous yellow and semitransparent integument.

In this study, we found that even after presoaking radicles in distilled water, a 2 - 4-day growth period after sowing in Petri dishes is necessary. After this period, radicles containing sufficient meristematic cells for cytological preparations are present in the samples.

Seeds germinated for 2 - 4 days in Petri dishes produced root tip of approximately 1 mm in length, and a higher frequency of cell divisions than meristems in the previous conditions. At this stage, the cotyledons were still closed and there were no secondary roots in the root tip.

In addition to obtaining meristems derived from newly germinated seeds in Petri dishes, it was possible to collect meristems from adult plants grown in pots containing soil and vermiculite, both in the vegetative and reproductive phases. In both cases, the meristems were suitable for cytogenetic analyses in *C. bonariensis*.

Meristems from adult plants, both in the vegetative (60 days after seeding) and reproductive phases (90 days after seeding), presented fewer cell divisions when compared to meristem from seeds germinated for 2 - 4 days in Petri dishes. According to [24], mitotic cell divisions in adult plant meristems are less frequent than mitotic cell divisions in newly germinated root tips.

In protocols described for *Conyza* genus [5] [20] root tips from seeds germinated in Petri dishes androot tips of adult plants [16] were used.

Regardless the stage of root apical meristem collection (from rootlets from newly germinated seeds and/or radicles from adult plants) the plants must be carefully handled, to avoid the breakage of meristem. The pots used for plant growth must be filled with soil and vermiculite (1:1) to facilitate the removal of the roots. Washing under running water facilitates the detachment of soil from the root. This procedure must be done carefully to avoid damage to the meristem.

The results of *C. bonariensis* antimitotic treatments are summarized in (**Table 3**). Regarding antimitotic treatments, our results indicate that the chemical antimitotic tested, in addition to cold water, do not effectively act in mitotic spindle depolymerization. The use of cold water as an antimitotic agent has the advantage that it is nontoxic. It has been reported to be effective for some species; however, it was not effective for *C. bonariensis* meristems (**Figure 1(a)**).

Similarly, cycloheximide was not efficient, causing chromosome clamping (**Figure 1(b)**). Besides this agglomeration, [28] reported that cycloheximide could cause chromosome supercondensation in the prophase and metaphase stages of some species. The results for colchicine were similar to the antimitotic effect of cycloheximide in terms of chromosome clamping, particularly at a concentration of 0.1% (**Figure 1(c)**).

Combinations of the agents 9-aminoacridine hydrochloride (0.05%) + 8-hydroxyquinoline (0.002 M) (**Figure 1(f)**), hydroxyurea (2.5 mM) (**Figure 1(h)**), and 8-hydroxyquinoline (300 ppm) + cycloheximide (**Figure 1(k)**)

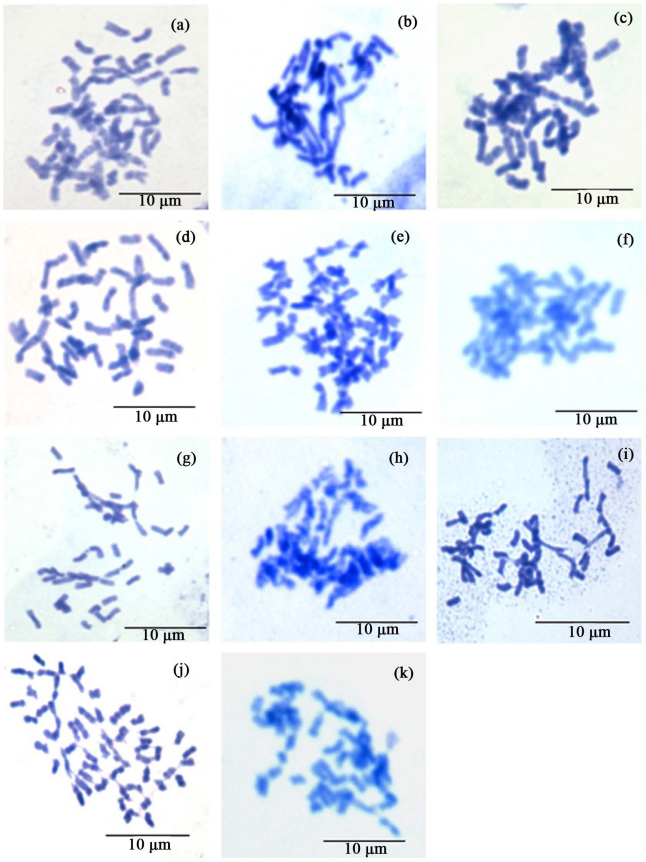

Figure 1. *Conyza bonariensis* mitotic metaphase cells (2n = 6x = 54) pretreated with different antimitotic agents. (a) Cold water; (b) Cycloheximide (6.25 ppm); (c) Colchicine (0.1%); (d) Colchicine (0.5%); (e) Esculin (0.04%); (f) 9-Aminoacridine hydrochloride (0.05%) + 8-hydroxyurea (2.5 Mm); (g) 9-Aminoacridine hydrochloride (0.05%) + para dicrhlorobenzene (saturated solution); (h) Hydroxyurea (2.5 mM); (i) Paradichlorobenzene (saturated solution); (j) 8-Hydroxyquinoline (0.02 M), (k) 8-Hydroxyquinoline (300 ppm) + cycloheximide (6.25 ppm).

Table 3. Concentrations and combinations of antimitotic agents used in the pretreatment of *Conyza bonariensis* root tips and their results.

	Antimitotic agents	Number of metaphase*
1	Cold water	7
2	Cycloheximide (6.25 ppm)	8
3	Colchicine (0.1%)	8
4	Colchicine (0.5%)	5
5	Esculin (0.04%)	8
6	9-Aminoacridine hydrochloride (0.05%) + 8-hydroxyquinoline (0.002M)	5
7	9-Aminoacridine hydrochloride (0.05%) + paradichlorobenzene (saturated solution)	3
8	Hydroxyurea (2.5 mM)	13
9	Paradichlorobenzene (saturated solution)	11
10	8-Hydroxyquinoline (0.02 M)	26
11	8-Hydroxyquinoline (300 ppm) + cycloheximide (6.25 ppm)	11

*Results over approximately 100 cells/pretreatment.

did not result in cells with well-spread chromosomes, and there was no stabilization and synchronization of the cells in the metaphase stage. Although these treatments resulted in a certain degree of condensation, chromosomes were in clusters with indefinite morphology. As a result, the primary and secondary constrictions could not be clearly observed, causing the counting and morphological chromosome analysis to be difficult (**Figure 1**).

Among the antimitotic agents evaluated, 8-hydroxyquinoline (0.002 M) treatment at 14˚C for 5 h was the only one that presented satisfactory results, as shown in (**Figure 1**, **Table 3**). In addition, pre-treatment with 8-hydroxyquinoline (0.002 M) was efficient for the roots of other species of the Asteracea family [29]-[32].

The use of 8-hydroxyquinoline as an antimitotic agent has been reported for *Conyza* species, usually at concentrations close to 0.002 M and incubation times ranging from 2 to 24 h [5] [16] [33]. In addition, there are reports of the use of 0.005% colchicine for 2 h [34] and cold water at 4˚C for 18 h [20].

With regard to the hydrolysis treatments tested, it was found that the hydrolyzing agent must be selected according to the type of radicle. Acid hydrolysis was effective for cell wall softening in radicles from newly germinated seeds cultivated in Petri dishes for 2 - 4 days. Enzymatic hydrolysis, however, was effective for cell wall softening of adult plants roots. Roots from seeds germinated for 2 - 4 days, when treated with the mixture of enzymes, were oxidized and presented darkened tips.

Roots from the seeds germinated in Petri dishes for 2 - 4 days and hydrolyzed for 6 - 10 min in 5N HCl at room temperature resulted in scattered cells with clear cytoplasm. Furthermore, the same result was observed when radicles from adult plans, both in vegetative (60 days after sowing) and reproductive phase (90 days after sowing), were incubated in the enzyme mixture for 60 min at 37˚C.

Researchers that have used acid hydrolysis with HCl 1 N at 60˚C for 10 min and HCl 2 N for 5 min, respectively, in *Conyza*; however, they do not mention the incubation temperature used in the process [16] [20].

Considering our results, it was determined that the root apical meristem of the *Conyza* genus can be obtained from both radicles from newly germinated presoaked seeds and young roots from adult plants. Both of these tissues provide material for cytological preparations throughout the plant cycle.

Pretreatment of meristems can be done using the same antimitotic agent, independent of the radicle development phase. Hydrolysis success appears to be dependent on the thickness of the cell wall, with acid hydrolysis being more effective for thin cell walls and enzymatic hydrolysis for thicker cell walls. Therefore, the choice of the hydrolyzing agent depends on the developmental stage of the meristem.

4. Conclusions

On the basis of this study, we concluded that:

The ideal stages for collection of root apex meristem of *C. bonariensis* are between 2 and 4 days after sowing the achenes in Petri dishes, and in the adult phase in plants, in both the vegetative and reproductive phases.

Antimitotic treatment with 8-hydroxyquinoline (0.002 M) at 14˚C for 5 h is the most efficient for the meristems, both from radicles of seeds germinated for 2 - 4 days and from roots of adult plants.

Hydrolysis with 5 N HCl for 6 - 10 min at room temperature is the most efficient for softening the radicle meristems from seeds germinated for 2 - 4 days.

Enzymatic hydrolysis for 60 min at 37˚C is the most efficient for softening the root meristems from plants in the adult stage.

We propose the use of these results as a basic technique for chromosomal preparations of *Conyza bonariensis*. This technique proposed can be used as a tool to obtain chromosomes in order to differentiate other *Conyza* species.

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