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## Development of tomatillo (*Physalis ixocarpa* Brot.) autotetraploids and their chromosome and phenotypic characterization

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The tomatillo, *Physalis ixocarpa* Brot. ( $2n = 2x = 24$ ), is an important crop in Mexico, and it is becoming appreciated in other countries. Polyploidy induction is expected to increase its breeding potential. The objective of this work was to develop and characterize tomatillo autotetraploids through colchicine-based induction. Young seedlings of the Rendidora cultivar were treated for 24 h with colchicine in concentrations ranging from 0.04% to 0.20%, and ploidy levels were tested by cytological and flow cytometry techniques. Autotetraploidy was induced with colchicine concentrations of 0.12% and 0.16%, with success rates of 67% and 65%, respectively. Presence of univalents, bivalents and multivalents was observed in prophase I and metaphase I. The basic genome size was not altered in the third generation progeny from treated plants. Autotetraploid plants were fertile and productive, but their pollen development was lower than their diploid counterpart. The polyploid plants showed higher values for life cycle length, plant height, fruit weight and equatorial diameter, fruits per plant, and soluble solid concentration. This is the first report of an autopolyploid cultivated tomatillo. Its genome duplication is readily induced with production of fertile plants, and may be valuable to introduce genetic plasticity in this crop.

**Key Words:** husk tomato, autopolyploid, fertility, meiotic pairing, pollen viability, plant size.

### Introduction

The cultivated tomatillo (*Physalis ixocarpa* Brot.) is a member of the Solanaceae family, and it is also referred as green or husk tomato. It was well known by Mayans and Aztecs before the arrival of Spaniards to Mexico, which is considered its putative domestication center. It produces a fruit that constitutes an important component of the Mesoamerican cuisine, and is employed in a similar manner to tomatoes (*Solanum lycopersicum* L.), but it has a slight acidic flavor. It is a source of vitamins A and C, and it has been suggested that chemicals present in tomatillos, e.g. ixocarपालactone A, may have cancer chemopreventive properties (Choi *et al.* 2006).

The husk tomato is an annual species, with a height from

1 to 1.5 m, adapted to humid tropical and subtropical conditions. The fruit is enclosed in a husk that usually breaks because of fruit expansion and has to be removed before consumption (Escobar *et al.* 2009). Flower budding occurs approximately 42 days after sowing, whereas flowering takes place at 48 days. Fruit development starts one week after flowering, and harvesting is carried out between 70 and 100 days after sowing (Mulato *et al.* 1987).

Both, the cultivated and most wild types of tomatillo, are diploid  $2n = 2x = 24$ , although some other species of the same genus are polyploids (Menzel 1951, Peña *et al.* 2002, Santiaguillo *et al.* 1994). It is an obligated outcrossing species that shows gametophytic self-incompatibility, which prevents the inbred line formation through self-pollination to obtain hybrids (Sahagún *et al.* 1999).

This species is native to Mexico and Central America, and it is presently one of the most important crops in Mexico (Cantwell *et al.* 1992), being the fourth vegetable in production surface with an area of 47,473 ha in 2009 (SIAP-SAGARPA 2010). This is due to its high consumption

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nationwide of 3.5 kg per capita, as well as its exportation to the United States of America and Canada. It is also cultivated in India, Australia and South Africa, as well as in the South Eastern United States of America. A wild species of tomatillo (*P. philadelphica*) has been adapted to some areas outside of its original range, including the South Eastern California (Peña *et al.* 2002). The average yield of tomatillo (*P. ixocarpa*) in Mexico is 13.933 t ha<sup>-1</sup>, a low quantity if we consider its potential yield estimated of 40 t ha<sup>-1</sup> (Peña and Santiaguillo 1999). Nonetheless, wide genetic variation exists in wild and cultivated tomatillos, which can be used for breeding purposes (Santiaguillo *et al.* 2004).

Autopolyploidy is an inducible biological state characterized by genomic redundancy, which can be capitalized by plant breeders. This characteristic increases the effective population size due to tetrasomic inheritance and enhances genome flexibility, thus facilitating the handling of selection. Also, genetic redundancy can potentially allow adaptive divergence of duplicated genes (Parisod *et al.* 2010). It has been demonstrated that autopolyploids are able to undergo rapid genomic changes, e.g. diploidization, showing high genomic plasticity (Doyle *et al.* 2008, Leitch and Leitch 2008). A possible drawback is fertility reduction; however, newly developed autopolyploids are highly variable for this characteristic, and often the reduction in fertility is very low (Ramsey and Schemske 2002). It is known that genome doubling is generally followed by changes in phenotypic traits like increase in cell size and modifications in ecological tolerance, although no consistent adaptive benefit is obvious (Parisod *et al.* 2010). Tomatillo is self-incompatible and that prevents inbred line formation to exploit hybrid vigor, polyploid induction may be an alternative to generate more vigorous plants without resorting to classical hybridization (Pandey 1957). Development of fertile autopolyploids in tomatillo would open the possibility of starting a new breeding strategy for this species, allowing the manipulation of yield and quality-related traits, as well as overcoming the drawbacks of its self-incompatibility.

The main objective of this work was to develop autopolyploid tomatillo plants from a high yielding cultivar, through chemical manipulation, as well as to perform cytological and phenotypic characterization of the products and their progenies.

## Materials and Methods

The parental material was the Rendidora cultivar, released in 1976 after four selection cycles from 49 local landraces from the State of Morelos in Mexico. It is an outstanding, high yielding cultivar, which still shows variation in growth habit, fruit color and size, among other traits. On average, it takes 90 days to reach maturity and the first flowers appear 28 to 30 days after sowing. Around 35% of its fruits show a diameter greater than 4.5 cm, and most of them completely fill the husk and show a firmness suited to commercialization. Fruits have an acid-sweet flavor, appreciated for sauce

preparation (Saray and Loya 1977).

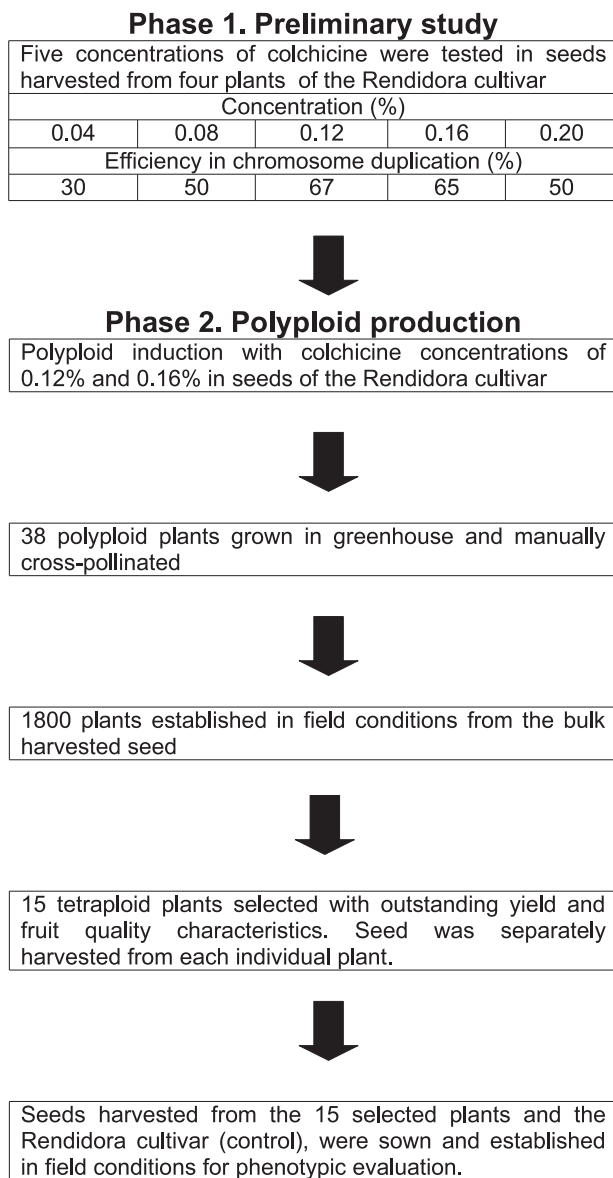
Polyploidy induction was carried out through the application of colchicine at concentrations of 0.04%, 0.08%, 0.12%, 0.16% and 0.20% on seeds harvested from four plants selected from the Rendidora cultivar based on yield and fruit size (Fig. 1). For each of the 20 treatments, 50 seeds were germinated in Petri dishes with 12 cm filter paper soaked with distilled water for 48 h at a temperature range of 23–26°C. Colchicine was applied when the seeds started to break their coats. After 24 h, the seeds were sown in 200-well polystyrene trays with peat moss. The developed plantlets were maintained on the trays until an approximate height of 12 cm, before being transplanted to an experimental field located in General Cepeda, in the State of Coahuila, Mexico.

At the beginning of the flowering stage, plants with leaves rounder, darker and bigger than the Rendidora type were chosen as polyploid candidates to evaluate the effect of colchicine through meiotic analysis. Considering the results of the first set of concentrations, the experiment was repeated with the best colchicine concentrations, being 0.12% and 0.16%. The newly obtained plants were grown in greenhouse conditions, and polyploid plants were identified through meiotic analysis. Afterwards, all of them were subjected to manual pollination to obtain the next generation consisting of 1800 plants, which were grown in an isolated plot. From this population, polyploid, high-yielding plants were selected and their progeny was established in the field, along the Rendidora cultivar, for replicated phenotypic evaluation (Fig. 1).

Planting of progenies across generations was carried out on 200-well polystyrene trays, filled with a 1 : 1 proportion of Canadian Sphagnum peat moss and perlite. Once the plantlets reached a height of 12 cm, with two pairs of true leaves, they were transplanted to the experimental field in rows separated by 1.80 m with an inter-plant distance of 60 cm. Nutrients were applied three times per week, with daily watering. N, P, K, Ca and Mg, in amounts of 2.40, 1.44, 1.47, 0.39 and 0.37 kg ha<sup>-1</sup>, composed the first solution respectively. From the 9<sup>th</sup> to the 15<sup>th</sup> week, the solution was changed to 1.90, 1.85, 2.75, 0.37 and 0.47 kg ha<sup>-1</sup>, respectively.

For meiotic analyses, flower buds were collected and fixed in a solution of 3 : 1 ethanol-glacial acetic acid for 24 h. Afterwards, the fixed flower buds were put on Petri dishes with distilled water to extract the anthers, which were set on a microscope slide with a drop of acetocarmine stain. Anthers were cut in halves with a dissection needle to liberate microsporocytes. After eliminating debris and allowing 2 min for staining, a cover slip was collocated and the slide was heated with alcohol flame and manually pressed.

As an additional check, mitotic chromosomes were prepared from root tips. An enzymatic technique originally applied to corn (Jewell *et al.* 1994) was adapted to tomatillo. Root apices from recently germinated seeds were treated with colchicine (0.25%) and fixed in 3 : 1 ethanol-acetic acid. Meristems were hydrolyzed with HCL 1N for 20 min at 60°C and then treated with a cellulase-pectolyase enzymatic



**Fig. 1.** Flow diagram of the process of autopolyploid development, from preliminary colchicine treatments to phenotypic evaluation in field conditions.

solution at 37°C for 50 min. For microscopic observation, meristems were dispersed with a curved needle on a slide and washed with the fixer solution 3 : 1 ethanol-glacial acetic acid. The cell material was stained with acetocarmine solution for microscopic observation. Both, meiotic and mitotic chromosomes, were analyzed on a compound microscope (Carl Zeiss, Heidenhem, Germany) and recorded by a digital camera (Pixera Viewfinder, New York) with the 100X objective lens. Around 15 cells were analyzed from each plant, to verify its ploidy level.

A flow cytometry check for polyploidy was done on a sample of 25 plants, developed from seeds harvested from five third-generation plants grown in the experimental field of General Cepeda (last part of Fig. 1). The cultivar

*Rendidora* was used as control. Approximately 50 mg of tissue material from young fresh leaves were placed on a Petri dish. Afterwards, 0.5 ml of stain (CyStain UV ploidy, Münster, Germany) was added to the tissue and this was chopped with a sharp blade. An additional amount of 1.5 ml of the same stain was added and incubated at room temperature for 5 minutes. The sample was filtered through a disposable filter (Partec 50 µm CellTrics, Münster, Germany). Finally, the cell suspension was analyzed in a flow cytometer (Partec PAII, Münster, Germany) using UV excitation and blue acquisition. Data was analyzed with the FloMax software (Partec, Münster, Germany), and peak analysis reports were generated for each sample.

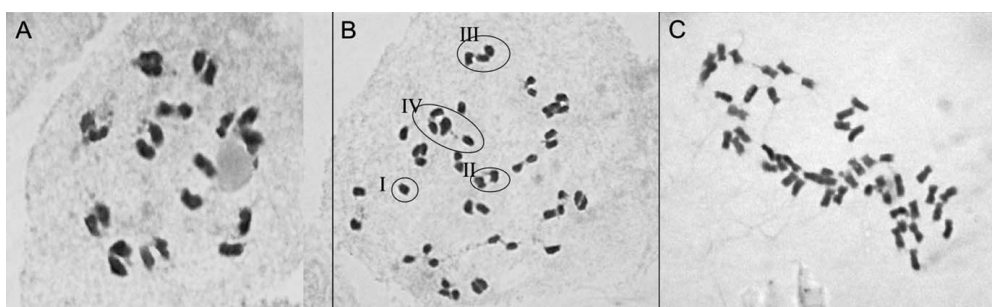
Pollen development was checked on the 15 second-generation selected polyploid plants and the *Rendidora* cultivar. Flowers were collected in the early morning previous to anthesis and placed on paper bags. Pollen grains were extracted in the laboratory, placed on glass slides and stained with acetocarmine 1%. Well-stained and round grains were considered fully developed, whereas those with poor staining and shrinkage were regarded as underdeveloped (Barcelos-Cardoso *et al.* 2004, Soares *et al.* 2008).

The progenies harvested from each of the 15 selected plants and the diploid *Rendidora* were comparatively characterized in a random complete block design with four replications and ten plants per plot. The following traits were evaluated: days to flowering, days to harvest maturity, plant height, stem diameter, fruit yield per plant, fruit size, soluble solid, and number of fruits per plant. Harvesting was carried out five times with 8-day intervals. Soluble solids in Brix degrees were measured with a precision refractometer (Carl Zeiss, Heidenhem, Germany). Comparisons between the group of autotetraploid progenies and the *Rendidora* cultivar were conducted for each evaluated trait through orthogonal contrasts in the language and environment for statistical analysis R within the analysis of variance procedure (R Development Core Team 2009).

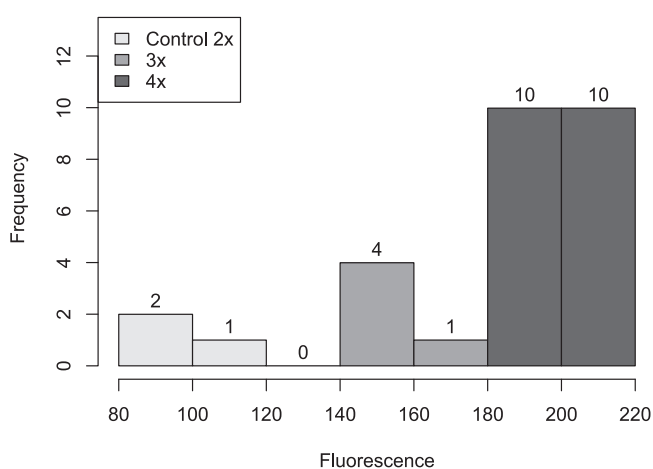
## Results

The 35 plants resulting from the first set of colchicine treatments and selected by phenotype, were composed by 14 diploid and 21 tetraploid individuals, revealed by meiotic analysis. The colchicine concentration of 0.04% gave a rate of success of 30%, whereas concentrations of 0.08% and 0.20% resulted in a rate of 50%. The greater efficiency in chromosome duplication was found with concentrations of 0.12% and 0.16%, giving success rates of 67% and 65%, respectively. With the next assay, using the two most efficient colchicine concentrations, 38 polyploid plants were obtained. From their bulked progeny of 1800 plants, 15 outstanding polyploids were selected (Fig. 1).

Meiotic analysis in diakinesis and metaphase I showed bivalent pairing for the *Rendidora* cultivar (Fig. 2A), while the autotetraploid plants exhibited pairing irregularities, with the presence univalents, bivalents, trivalents and



**Fig. 2.** Chromosomes of *P. ixocarpa*. A. Diakinesis of diploid tomatillo from the Rendidora cultivar, showing 12 bivalent configurations. B. Diakinesis of a colchicine-induced tetraploid of tomatillo showing seven univalents, 11 bivalents, five trivalents and a putative tetravalent. An example of each type of configuration is marked on the photograph. C. Mitotic metaphase of a colchicine-induced tetraploid of tomatillo showing a chromosome number  $2n = 4x = 48$ .



**Fig. 3.** Distribution of the main peak of relative fluorescence for control plants (Rendidora) and progenies of tetraploid plants of *P. ixocarpa*.

quadrivalents (Fig. 2B). Mitotic analysis of the progenies of the 15 second-generation selected plants, grown in field conditions, confirmed their tetraploid status, showing in all cases chromosome complements with  $2n = 4x = 48$  (Fig. 2C). Furthermore, flow cytometry was applied to 25 progenies of five random plants cytologically identified as tetraploids. From them, 20 plants were found to be tetraploid, whereas five plants showed peaks indicating a triploid condition. In the three tested plants, the control cultivar Rendidora showed profiles indicating a diploid condition. The distribution of the main fluorescence peak for flow cytometry in the 25 experimental plants and the diploid control group showed a clear-cut distinction among materials with 2x, 3x and 4x conditions (Fig. 3).

Pollen development in the tetraploid progenies had a value of 61.85%, whereas Rendidora showed an estimate of 82.52% (Table 1), and the difference was statistically significant.

Tetraploid plants showed significantly greater values of days to flowering, days to harvesting, fruit equatorial diameter, fruits per plant, plant height and soluble solid concentration, as compared with the Rendidora cultivar. The life cycle traits days to flowering and days to harvest were in-

**Table 1.** Comparative traits between the Rendidora cultivar ( $2n = 2x = 24$ ) and tetraploid plants ( $2n = 4x = 48$ ) of *P. ixocarpa*

Trait	Rendidora	Tetraploid
Days to flowering	44.15	52.85***
Days to harvesting	80.25	89.75***
Fruits per plant	62.64	53.07***
Fruit polar diameter (mm)	35.27	34.06NS
Fruit equatorial diameter (mm)	42.13	48.60***
Fruit weight (g)	33.67	38.14**
Soluble solids ( $^{\circ}$ Brix)	6.06	6.78*
Pollen viability (%)	82.52	61.49**
Stem diameter (cm)	2.36	2.50NS
Yield per plant (kg)	1.65	2.055NS
Plant height (cm)	82.19	90.62*

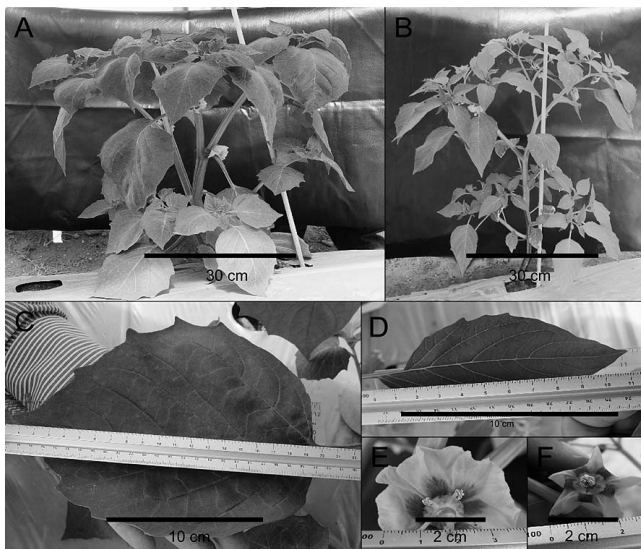
\*, \*\*, \*\*\* Significant at the 0.05, 0.01 and 0.001 probability levels, respectively.

NS Non significant.

deed significantly longer in the tetraploid plants than in their diploid counterpart. However, fruit yield per plant was not significantly different (Table 1). Polyploid plants were easily identifiable by their greater leaf and flower sizes (Fig. 4).

## Discussion

The use of colchicine for polyploid induction in tomatillo was very effective within the range of concentrations from 0.12 to 0.16%, applied to young seedlings during 24 h, with an approximate rate of success of 66%. Although meiotic irregularities were found, the reduction in pollen development did not hinder plants from producing fruits and viable seeds. There is an expected increase of bivalent pairing through generations, as it was observed in *Arabidopsis* (Santos *et al.* 2003). In fact, established autotetraploids show remarkable genome reorganization, like functional diploidization and significant decrease in chromosome length, as compared with newly synthesized ones (Parisod *et al.* 2010). For pollen viability, it was already mentioned that this trait might not be strongly affected by autopolyploidy (Ramsey and Schemske 2002). Flow cytometry did not show DNA reduction in third-generation polyploid plants (Fig. 2). However,



**Fig. 4.** Comparative phenotypes of tetraploid and diploid plants of *P. ixocarpa*. A. Colchicine-induced tetraploid. B. Diploid control Rendidora. C. Leaf of a tetraploid plant, showing a length of 20 cm. D. Leaf of a diploid plant, showing a length of 11 cm. E. Flower of a tetraploid plant, showing a diameter of 4 cm. F. Flower of a diploid plant showing a diameter of 3 cm.

this phenomenon has been observed in advanced generations of duplicated genomes, as a part of its reorganization (Rivero-Guerra 2008), and it is likely that it will occur later on in the progeny of these tomatillo polyploids.

The appearance of some triploid plants among the seeds harvested from second-generation plants in the experimental field in General Cepeda, a rural area in the south of Coahuila, could have been caused by exogenous pollen contamination. In fact, we have recently detected the presence of wild plants of *P. ixocarpa* in the same area, which may have donated the pollen that gave raise to triploid plants. Another possibility is the production of some gametes with the basic chromosome number from the tetraploid plants. However, pollen contamination did not affect previous generations, since they were handled in isolated plots.

Phenotype differences between diploid and tetraploid plants, allowed identification by their visual appearance at early stages, because the leaves of polyploids looked markedly larger. Later on, flowers in polyploid plants also showed a greater size. Also, the results indicate a longer life cycle than their diploid counterparts. The fact that soluble solids and fruit equatorial diameter showed significantly higher values in tetraploids, is a promising result in terms of breeding potential, even though fruit yield was not significantly different between tetraploid and diploid plants.

The successful induction of tomatillo (*P. ixocarpa*) autotetraploids through induction by colchicine in this work, is the first case reported in the literature. Their easy production, new traits and fertility, indicate that use of autopolyploidy in *P. ixocarpa* can serve as a basis of a novel tomatillo breeding program.

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